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Quantitative contrast harmonic imaging of the normal and diseased canine spleen

INAUGURAL-DISSERTATION

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

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Zürich 2007

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1. Summary

Splenic lesions are common in dogs and easily detected ultrasonographically, but their appearance usually does not allow a definitive diagnosis. The purpose of the present study was to evaluate ultrasonographic perfusion patterns and quantify perfusion in the normal and diseased canine spleen by the use of a new ultrasonographic technology, contrast harmonic imaging.

Of 24 dogs, 13 spleens were considered normal and 11 spleens abnormal. Cytological or histological diagnosis was hemangiosarcoma (n=3), hyperplasia of the lymphatic tissue (n=4), extramedullary hematopoiesis (n=1), malignant lymphoma (n=1) and splenic torsion (n=1). The spleen of another dog was normal sonographically; reactive hyperplasia was found cytologically.

A protocol was established for the examination of the spleens with contrast harmonic imaging. The perfusion pattern in normal spleens was described. Benign changes of the spleen enhanced well. In the hemangiosarcomas, enhancement was very low. In the diffuse malignant lymphoma, lesions enhanced similar to normal splenic tissue. No enhancement was seen in the splenic torsion. Quantitative assessment of perfusion revealed similar results; however, statistical analysis was not yet significant.

In conclusion, perfusion appears to differ among normal splenic tissue and benign and malignant lesions. Contrast harmonic imaging may represent a useful non-invasive tool to assess splenic lesions in the dog. More cases need to be investigated to prove the results of the present study.

2. Introduction

2.1. Diseases of the spleen in dogs

Morphological changes of the spleen are common in the dog. They include circumscribed focal masses or diffuse changes with or without overall splenic enlargement (splenomegaly). Splenic masses can be classified as neoplastic or non-neoplastic. Neoplastic splenic masses again include benign and malignant lesions (Couto and Hammer 1995). The pathological classification of splenic diseases in the dog is rather inconsistent in the literature. Moreover, a variety of terms are used interchangeably by some authors such as nodular hyperplasia, lymphoid hyperplasia and nodular lymphoid hyperplasia, as well as lymphoplasmocytic splenitis and lymphoreticular hyperplasia. However, this is in disagreement with other authors. The following summary is mainly based on the classification by Jubb et al. (1993) which includes developmental, degenerative, circulatory, inflammatory, neoplastic and benign hyperplastic diseases as well as hematoma, rupture, torsion and cysts (Searcy 2001, Valli 1993). Allowance was made to important aspects of literature by other authors.

2.1.1. Developmental diseases

Developmental diseases include congenital absence, congenital ectopia, hypoplasia, shape anomalies, congenital or acquired accessory spleens, duplication and ectopic pancreatic tissue (Trautwein 1991, Valli 1993).

2.1.2. Degenerative diseases

Degenerative diseases are often seen in older dogs, such as senile atrophy of the spleen or siderotic nodules/plaques (Jones and others 1997, Searcy 2001, Valli 1993). The latter represent deposits of iron and calcium in connective tissues. The salts become encrusted and are of a yellowish color. In association with ceroid, these nodules likely represent the residual effects from areas of hemorrhage. Their deposition in the capsular margins and internally

suggests that a dystrophic or storage phenomenon may be more likely (Valli 1993).

Hyperimmune states and stress may also lead to degenerative changes such as lympholysis, epithelioid and hypocellular appearance of the germinal centers, and a variety of vascular changes which may proceed to mineralization (Valli 1993).

Amyloidosis represents a rare degenerative process in the spleen, occurring as a part of generalized amyloidosis. It involves the germinal centers and the enlarged and waxy follicles protrude from the cut-surface giving the organ the sago-spleen appearance (Searcy 2001, Trautwein 1991, Valli 1993).

Hemosiderin is a storage form of iron. The amount and form of storage iron varies greatly in normal animals with age. Hemosiderosis represents an excessive amount of hemosiderin due to hemolytic anemia causing tissue injury and fibrosis. Rare storage diseases of the spleen have been described in the dog such as sphingomyelin storage in the nervous system and the spleen (Searcy 2001, Trautwein 1991, Valli 1993).

2.1.3. Circulatory disorders

There are various circulatory disorders that cause splenomegaly. Passive congestion occurs most commonly due to the use of barbiturate drugs or acepromazine which cause relaxation of the smooth muscle of the splenic capsule. With propofol, no enlargement was found in a recent study (O'Brien and others 2004b). Cardiac, pulmonary or portal venous congestion does not appear to be as common as in humans causing congestion of the spleen in the dog. This may be due to the highly muscular capsule which is not easily distended (Valli 1993), or due to the development of velar-omental veins that decompress the spleen by shunting blood to the left renal vein (Couto and Hammer 1995). Torsion of the spleen either isolated or in association with the gastric dilatation-volvulus syndrome, commonly results in a marked splenomegaly caused by congestion due to occlusion of the splenic vein. Ultimately, occlusion of the arteries occurs, resulting in ischemia, which is followed by hemolysis and

diffusion of hemoglobin through the devitalized splenic capsule. This process results in hemoglobinemia and hemoglobinuria (Searcy 2001, Valli 1993). Most often affected are dogs of large, deep-chested breeds. Clinical signs can be either acute or chronic.

Thrombosis of the splenic vein has been observed in association with splenic congestion of any cause, hypercoagulable states and hyperviscosity syndrome (Couto and Hammer 1995). Embolism with infarction is also not uncommon, is mainly seen in the splenic artery and usually derives from valvular vegetations (Couto and Hammer 1995, Valli 1993).

Active hyperemia may be seen with acute systemic infections (Valli 1993).

2.1.4. Inflammatory changes

Inflammatory changes within the spleen usually result in diffuse enlargement of this organ. Most disorders associated with splenitis are infectious in nature. In addition to lymphoreticular hyperplasia, splenic changes in splenitis include hematogenous infiltration with inflammatory cells. According to the course of the disease, splenitis can be classified as either acute, subacute, or chronic; according to the predominant cell type, they can be classified as suppurative (predominant cell type: the neutrophil), necrotizing (necrosis predominates), eosinophilic (eosinophils predominate), lymphoplasmocytic (lymphocytes and plasma cells predominate), granulomatous (macrophages, epithelioid cells, lymphocytes, or multinucleated giant cells predominate), and pyogranulomatous (granulomatous changes and neutrophilic infiltration are present) (Searcy 2001). The terms lymphoplasmocytic splenitis and lymphoreticular hyperplasia are frequently used interchangeably. It is important to classify the splenitis according to the predominant cell type, since different etiologic agents are associated with the different forms. Diseases associated with suppurative splenitis include penetrating wounds, migrating foreign bodies, hematogenous dissemination of bacterial infections, bacterial infections secondary due to splenic torsion, protozoal infections (toxoplasmosis), and certain viral diseases. If cavitated lesions form with suppurative

splenitis, the term splenic abscess is preferred. Necrotizing splenitis may be caused by gas-forming anaerobes, seen with splenic torsion and neoplasia e.g. lymphoma, hemangiosarcoma or histiocytic sarcoma.

Eosinophilic infiltrates have been observed with eosinophilic gastroenteritis in the dog. Lymphoplasmocytic splenitis occurs in association with subacute or chronic infectious disorders. Granulomatous splenitis occurs in some systemic mycosis and mycobacterial infections (Searcy 2001).

2.1.5. Benign hyperplastic diseases

Benign hyperplastic changes of the spleen are composed of cells normally found in the canine splenic parenchyma (Spangler and Kass 1998). Nodular hyperplasia is frequently observed in the spleen of old dogs; together with splenic hematomas, they made up most of the splenic lesions in a very large study group (Spangler and Culbertson 1992). Hyperplastic nodules lack a capsule and germinal centers, consisting of monomorphic medium or large lymphocytes with moderate cytoplasmic volume and small nucleoli. There may be many mitoses, and the lesions are judged to be benign more on knowledge of behavior than cytology. There is no evidence to suggest that these lesions ever progress to lymphoma (Valli 1993). Microscopic examination of splenic nodular hyperplastic lesions often reveals varying and sometimes complex cellular composition. These nodules can thus be grouped into distinct morphologic categories based on the relative proportion and type of cellular components that make up the nodule (i.e. lymphoid, hematopoietic, plasmacytic, or mixed) (Spangler and Kass 1998).

Splenic plasmacytoma is a rare condition in dogs. Cytologically, the cells are uniform and of moderate size but with abundant, usually eccentric cytoplasm. Nucleoli are small but prominent and central, and there is mild heterochromatin aggregation. Mitoses are less numerous than in the normal surrounding tissue. Plasmacytoma may progress to an immunoblastic type of lymphoma within a year of biopsy (Valli 1993).

Hematopoietic alterations include extramedullary hematopoiesis, myeloid metaplasia, myelolipoma, and lymphoid hyperplasia (Valli 1993). Extramedullary hematopoiesis is common in dogs. The spleen retains its fetal hematopoietic potential during adult life. A variety of causes may stimulate the spleen's fetal hematopoietic function and producing red blood cells, white blood cells, and platelets (Searcy 2001). Megakaryocytes are the most obvious hematopoietic precursors and characteristically lie adjacent to the smooth muscle trabeculae when stimulation is mild, but may become diffusely distributed in the sinus areas when stimulation is prolonged and pronounced. In contrast, myeloid metaplasia is a condition where there is diffuse hematopoiesis in the spleen without an apparent target for the increased cell production (Spangler and Kass 1999, Valli 1993).

Myelolipomas are composed of normal marrow with about 50% hematopoietic and the rest, large typical fat cells (Valli 1993).

Lymphoid hyperplasia of the spleen occurs by expansion of the periarteriolar lymphoid sheaths (Valli 1993).

In the present study, the term reactive hyperplasia is also used. The clinical pathologists at our faculty preferably use it to describe reaction of the white pulp with an increased number of medium-sized to large lymphocytes and plasma cells. If the extent of reactive hyperplasia is less than 10%, then it may be considered normal splenic tissue.

Hyperplastic nodules of the spleen also include a distinct population of spindle and/or histiocytic cells (fibrohistiocytic cells) that in some cases displace or distort the lymphoid follicular architecture of the nodule. This type of nodule seems to represent the benign limit of a continuum between nodular hyperplastic proliferation in the splenic parenchyma and nodular neoplasms consisting of pleocellular components (malignant fibrous histiocytoma). However, malignant fibrous histiocytoma exhibits a broad range of biological behavior (Spangler and Kass 1998).

2.1.6. Hematoma

Splenic hematomas are very common in dogs, and the cause is often not obvious. They may occur secondary to trauma. Most dogs are relatively healthy (Searcy 2001). However, they may also undergo spontaneous splenic rupture and hemoabdomen (Prymak and others 1988). One report describes the relation of splenic hyperplastic nodules and splenic hematomas in a very large group of samples. The authors observed superimposition of nodular lymphoid elements with hematomas. They suggest that with nodular lymphoid hyperplasia, marginal zone distortion with regional disruption of splenic blood flow is seen, leading to failure of marginal zone circulation and accumulation of blood within and around the hyperplastic nodule. Eventually, this causes hematoma formation, hypoxia and necrosis (Spangler and Culbertson 1992). Splenic hematomas may also be associated with neoplastic lesions such as hemangiomas, hemangiosarcomas or lymphomas (Jones and others 1997).

2.1.7. Rupture

Rupture of the spleen is not uncommon in the dog and caused by a major trauma such as a car accident. Bleeding to a various degree is found and fragmentation of the spleen may be seen. Neoplastic diseases such as hemangiosarcoma or lymphosarcoma may also lead to rupture of the capsule and exsanguination. Pathologic rupture of the spleen may also occur with congestive, hemolytic or infectious diseases (Jones and others 1997, Valli 1993).

2.1.8. Cysts

Splenic cysts are unusual and they may occur in conjunction with infection with *Echinococcus* species, cystic degeneration of hematomas, or neoplastic cysts such as hemangiomas or hemangiosarcomas (Jones and others 1997, Valli 1993).

2.1.9. Neoplastic diseases

Neoplastic splenic masses include benign and malignant lesions and are represented mainly by endothelial tumors, namely hemangiomas and hemangiosarcomas. Other neoplasms that may present as splenic masses in dogs are fibrosarcomas, leiomyosarcomas, leiomyomas, malignant fibrous histiocytomas, malignant histiocytosis, lymphomas and mesotheliomas (Couto and Hammer 1995, Cruz-Arambulo and others 2004, Searcy 2001, Spangler and Kass 1998). It appears that splenic masses are more common than diffuse splenic enlargement in dogs, whereas the opposite occurs in cats (Searcy 2001). Neither the size nor the gross appearance of splenic masses can be used as a guide to determine their biological behavior. It is not uncommon to observe extremely large hematomas whereas hemangiosarcomas may be small. Moreover, hepatic extramedullary hematopoiesis or regenerative hepatocellular hyperplasia may result in the appearance of hepatic masses that mimic metastatic lesions, even in dogs with splenic hematomas (Couto and Hammer 1995).

Generalized enlargement of the spleen with diffuse infiltration with neoplastic cells is a common finding in dogs with lymphoma, mastocytosis, malignant histiocytosis, multiple myeloma, and is rarely seen with splenic metastases from mammary adenocarcinomas and intra-abdominal (e.g. pancreatic) carcinomas (Couto and Hammer 1995). Splenic metastases may also appear as focal masses.

Hemangiosarcomas appear to be extremely common in dogs and represent the most frequent neoplasm of the canine spleen (Spangler and Culbertson 1992). However, the same authors found that the combined prevalence of all other splenic neoplasms (nonangiomatous and nonlymphomatous) was similar to that of the hemangiosarcoma alone. Hemangiosarcomas occur predominantly in older large-breed dogs, and there is a predilection for males and for German shepherd dogs as well as for Golden Retrievers. Clinical sings and physical findings in dogs with splenic hemangiosarcomas are usually vague and non-specific. They include anorexia, weight loss, abdominal distension, weakness, pallor and vomiting. A large majority

of dogs with hemangiosarcomas are examined because of rupture of the primary tumor (Couto and Hammer 1995). Hemangiosarcomas have aggressive biological behavior. More than 50% of the affected dogs have gross evidence of metastatic disease on initial presentation which is usually found in the liver, omentum, peritoneum, kidneys, heart and lungs (Couto and Hammer 1995). Surgical resection has been the mainstay of therapy in dogs with splenic hemangiosarcoma. In one study, splenectomy in dogs with hemangiosarcoma resulted in a median survival of 65 days (mean 80 days). Depending on the protocol, median survival may be improved as a result of chemo- or immunotherapy (Couto and Hammer 1995).

Compared to hemangiosarcomas, hemangiomas behave biologically not aggressive. They are not as common and depending on the study, they represent 1-10% of splenic masses. Most dogs with splenic hemangiomas are clinically healthy and rupture with abdominal bleeding is rarely seen (Couto and Hammer 1995, Searcy 2001).

Malignant histiocytosis is characterized by malignant histiocytes, which have marked cellular atypia. Another common finding is the presence of bizarre multinucleated giant cells (Vail 2001). Malignant fibrous histiocytoma is characterized by cells with variable fibroblastic to less obviously histiocytic cells and associated variable numbers of nonneoplastic inflammatory cells (Hayden and others 1993, Kerlin and Hendrick 1996). Some histiocytic neoplasms have characteristics of both malignant histiocytosis and malignant fibrous histiocytoma. These neoplasms have been defined as histiocytic sarcomas. In the dog they include tumors of macrophage and tumors of dendritic cell origin which resemble dendritic cell sarcomas described in humans. Such tumors are rapidly growing and locally aggressive. The prognosis, despite treatment, is poor with an average survival time of 5.3 months and a high metastasis rate (Craig and others 2002). Malignant histiocytosis and malignant fibrous histiocytoma most commonly affect the lung, the hilar lymph nodes, the mesenteric lymph nodes, the liver and the spleen. Bernese mountain dog, Golden Retriever, Rottweiler, and Doberman pinscher breeds have been reported to be at higher risk for malignant histiocytosis,

whereas malignant fibrous histiocytoma has been reported most commonly in Golden Retrievers. The etiology of malignant histiocytosis is unknown. However, familial occurrence has been reported in Bernese mountain dogs, suggesting the possibility of a genetic predisposition. Prognosis is grave and response to combination chemotherapy has been generally unrewarding, at best resulting in short-term palliation (Vail 2001).

2.2. Diagnosis of splenic diseases in dogs

During a routine physical examination, the spleen can be palpated in the dog as a flat structure oriented dorsoventrally in the left anterior abdominal quadrant. However, it has to be emphasized that palpably enlarged spleens are not always abnormal, and that enlarged spleens are not always palpable. Clinical and physical signs of dogs with a focal or generalized enlarged spleen are usually vague and nonspecific. With the exception of splenic torsion, they are in general related to the primary disease rather than to splenic enlargement per se (Couto and Hammer 1995).

2.2.1. Radiographic imaging

The spleen is normally well visualized on abdominal radiographs, but there is a wide variation in its appearance. Radiographically, the spleen is an elongated, flat, solid organ in the cranial left abdomen, lying just caudal to the stomach. The dorsal extremity of the spleen (head) is less variable in its position because of the short gastrosplenic ligament and its location between the gastric fundus and the cranial pole of the left kidney. There, the head is seen as a triangular structure. The ventral extremity is less tightly fixed in position and thus may vary greatly in location. Because determination of normal splenic size is subjective, alterations in the size of this organ must be substantial to be recognized. The spleen may increase in size uniformly and diffusely, or the spleen may enlarge locally, as with a splenic mass, with or without alteration in the splenic shape and margination. Gas accumulation in the splenic pulp

is seen rarely and may be associated with necrosis, splenic torsion and proliferation of gas-forming organisms (Pechman 1998).

2.2.2. Ultrasonographic imaging

The size of the spleen is variable and must be assessed subjectively, similar to radiography. The capsule is smooth, regular and echogenic when the beam strikes it perpendicularly. With splenic masses, the capsule is interrupted or deviates outward, and the splenic parenchyma is continuous with the mass. The normal splenic parenchyma is homogeneous with a finely textured, medium to high level echo pattern. This pattern contrasts with the coarse slightly less echogenic appearance of the liver. The spleen is normally considerably more echogenic than the cortex of the left kidney. The splenic arterial branches are difficult to see on routine splenic scans. Doppler ultrasonography can help determine their location. The branches of the splenic vein are easily detected near the hilus of the spleen, but their course into the splenic parenchyma can be followed only for a short distance in the normal animal. The size of the splenic veins must be judged subjectively. The main splenic vein can usually be traced from the spleen to the main portal vein. The splenic hilar region should always be carefully searched for lymphadenopathy (Nyland and others 2002a).

With diffuse splenic diseases (infectious, inflammatory, non-neoplastic/non-inflammatory, benign or malignant neoplastic), hypo-, hyperechogenicity or normal echogenicity may be seen in combination with or without diffuse splenomegaly. Ultrasonography is not helpful in establishing a specific diagnosis and the differential diagnosis list is long. Therefore, a biopsy is usually indicated unless the diagnosis is obvious from other clinical information (Nyland and others 2002a). In a recent study it was not only shown that significant enlargement of the spleen occurs due to the use of barbiturate drugs or acepromazine, but attenuation and backscatter was also markedly increased after the administration of acepromazine. With propofol, no enlargement was found (O'Brien and others 2004b).

Splenic torsion produces a markedly enlarged spleen with diffuse anechoic areas (lacy pattern) and multiple parallel echogenic lines within the parenchyma. The anechoic areas are thought to represent dilated sinusoids from splenic congestion, whereas the parallel echogenic lines are thought to be severely dilated vessels. A lacy pattern without dilated vessels (parallel echogenic lines) may also be seen with acute splenitis, necrosis and infarction from other causes than torsion (Mai 2006, Nyland and others 2002a). The splenic veins near the hilus are also enlarged from venous outflow obstruction. Infarction, necrosis, abscess formation, thrombus formation in the splenic veins or free abdominal fluid may be seen. Doppler evaluation of the splenic veins indicates an absence of blood flow. Arterial flow may or may not be present, depending on the degree of torsion (Saunders and others 1998). However, absence of flow might also be due to splenic vein thrombosis, even when intraluminal echoes cannot be identified (Mai 2006). The presence of a hilar hyperechoic perivenous triangle was significantly associated with splenic torsion in a recent study. This sign was continuous with the hyperechoic mesentery and although not being pathognomonic, it may be associated with acute splenic torsion because of the secondary severe diffuse splenic enlargement (Mai 2006). Focal lesions of the spleen are easily detected, but the ultrasound appearance usually does not allow a definitive diagnosis. Hyperplastic nodules, hematoma, abscess, primary or metastatic neoplasia, necrosis from vascular compromise, toxic conditions and inflammatory disorders can produce similar ultrasound findings. Depending on its age, a hematoma is extremely variable. Intraparenchymal hemorrhage may initially appear hyperechoic but larger collections of unclotted blood are initially hypoechoic to anechoic. Clotted blood may appear iso- to hyperechoic. A cystlike structure may develop as the clot organizes. Hematomas and infarcts generally get smaller over time. Infarcts may show a hypoechoic, wedge-shaped appearance with the base towards the splenic margin. However, they may also appear poorly marginated, circular in shape or as complex lesions. An abscess is likely if intense areas of hyperechogenicity with dirty shadowing are present within the lesion because of gas-forming

microorganisms. Hyperplastic nodules are not always observed ultrasonographically. They may only alter the otherwise smooth margin of the spleen, may be iso- to hypo- or even hyperechoic in comparison to the normal spleen or may even have a complex appearance. The type of malignant splenic neoplasia can neither be determined from its ultrasonographic appearance. Even cavitated lesions are not pathognomonic of hemangiosarcoma (Nyland and others 2002a).

2.2.3. Computed tomography and magnetic resonance imaging

In humans, ultrasound largely has been replaced by computed tomography (CT) and magnetic resonance imaging (MRI) for the characterization of focal hepatic and splenic lesions. So far, there are only two reports in the literature describing the use of CT and MRI for the diagnosis of splenic lesions in the dog.

In a recent study, the authors investigated the use of CT for the differentiation of splenic masses (Fife and others 2004). Malignant splenic masses had significantly lower attenuation values measured in Hounsfield units, than non-malignant splenic masses, on both pre- and post contrast images. On post-contrast images, there was a significant difference in attenuation characteristics among the three subset of masses (malignant, hematoma, hyperplasia), with nodular hyperplasia having the highest Hounsfield units, hematomas having intermediate Hounsfield units, and malignant masses having the lowest Hounsfield units values. A receiver operator characteristic curve of post-contrast medium Hounsfield units revealed 55 as the best threshold value to distinguish malignant from nonmalignant masses, with those less than the threshold value being malignant. The authors suggested that the low Hounsfield units for the hemangiosarcomas were due to the formation of large hematocysts or blood-filled cavities. The high degree of contrast enhancement of the hyperplastic nodules may have been related to the high degree of vascularity of hyperplastic lymphoid tissue. The underlying etiology of splenic hematomas in the dog was thought to

explain the intermediate degree of contrast enhancement. In contrast to humans, splenic hematomas in the dog are often secondary to a primary disorder such as nodular hyperplasia. None of the dogs in their study had a history of trauma. However, the malignant masses in that study mainly included hemangiosarcomas and only one fibrosarcoma and one malignant fibrous histiocytoma. The nonmalignant group included dogs with splenic hematoma, nodular hyperplasia and one nonmalignant fibrohistiocytic nodule (Fife and others 2004).

In human patients, the inherently high soft tissue contrast of MRI allows the differentiation of benign and malignant hepatic and splenic lesions. In a recent study in dogs, a MRI classification system similar to humans was used successfully on the basis of lesion signal characteristics to differentiate malignant from benign splenic lesions in dogs. Malignant lesions showed very similar imaging characteristics in both humans and dogs (Clifford and others 2004).

2.2.4. Cytology and histology

The spleen is easily accessible with ultrasound and ultrasound-guided biopsies are easily performed. Aspiration procedures are most useful in detecting lymphoma or mast cell involvement. Tissue-core biopsies have been avoided because of a high potential for hemorrhage although some authors reported a lower complication rate and conclude that tissue-core biopsies of solid splenic masses are useful and can be performed safely. Lower quality samples may be attributed to red pulp congestion because of anesthesia or engorgement of the spleen. Aspirates and tissue-core biopsy specimens of complex splenic masses primarily containing fluid-filled cystic structures (e.g. hemangiosarcomas) are less useful and often non-diagnostic because of blood dilution (Nyland and others 2002b). Therefore, a negative aspirate does not rule out neoplasia. Moreover, repeated hemorrhages, necrosis, and organization may make hemangiosarcomas difficult to distinguish from longstanding infarction of the spleen or hematomas (Nyland and others 2002a). The critical

item in the diagnosis of hemangiosarcoma is the presence of typical vascular-forming malignant endothelial cells (Jones and others 1997).

2.3. Contrast harmonic imaging

The concept of contrast imaging was introduced to ultrasound almost 30 years ago. It was first observed in echocardiography, rather incidentally, when small air bubbles were noted surrounding a catheter tip placed in the left ventricle during cardiac catheterization producing transient high reflections (Gramiak and Shah 1968). From that time on, intensive research was dedicated to the development of clinically relevant ultrasound contrast agents. Technical developments enabled the production of microbubbles that are stable in the circulation, traverse the pulmonary circulation to allow recirculation, and are inert to the patient. These dramatic improvements in ultrasound contrast agent persistence and efficacy have led to the extended usefulness of ultrasound imaging in human medicine and clinical research. Similarly, the advent of microbubble contrast agents has encouraged the development of new ultrasound technologies such as specific contrast imaging sequences. These developments have brought new possibilities such as assessment of tissue perfusion into clinical application and research.

2.3.1. Properties of ultrasound contrast agents

An ultrasound contrast agent is an exogenous substance, consisting of gas or air microbubbles encapsulated by a shell of different composition, that can be administered intravenously or into a body cavity to enhance ultrasonic signals. Commercially available microbubble contrast agents are approximately 2-6 μm in diameter; they are much smaller than the wavelength of the ultrasound beam (approximately 0.5 mm in water at 3 MHz) and therefore, behave as scattering reflectors. Because gases have acoustic impedance very different from blood and tissue, microbubble contrast agents have a good scattering yield (Dalla Palma and Bertolotto

1999). Although highly reflective, these agents also are highly attenuating at fundamental frequencies, causing severe loss of signal strength as the sounds travels through the enhanced portions of the body. Various studies have documented the unacceptably high attenuation coefficient which is directly frequency-dependent at fundamental transmitted frequencies (Couture and others 2006).

Most ultrasound contrast agents do not diffuse across the endothelium and therefore, they basically are blood pool agents. Vascular enhancement usually lasts a few minutes. Typically, the gas content of the contrast agent is eliminated via the lungs, while the shell components are filtered by the kidney and eliminated by the liver (Quaia 2005a).

Some agents have additional tissue specific properties. After blood pool clearance, Levovist has been shown to have a late hepatosplenic-specific parenchymal phase with accumulation in the human liver and spleen up to 20 min after intrvenous bolus injection. The underlying mechanism is not fully understood; accumulation may be mediated by the reticuloendothelial system, e.g. the Kupffer cells, or microbubbles are entrapped in the liver sinusoids (Quaia 2005a). Therefore, the late phase is also called sinusoidal phase. Other agents such as SonoVue or Imavist, although primarily designed as blood pool agents, were also subsequently found to have the sinusoidal phase because the agents are believed to be trapped or slowed in the hepatic sinusoids (Leen and Horgan 2003). Another study reported spleen-specific parenchymal uptake of SonoVue in humans (Lim and others 2004).

2.3.2. Principles of action

The behavior of the microbubble under ultrasound beam is governed by parameters such as resonance frequency, pulse repetition frequency, number and location of focal zones, acoustic power, the filling gas, damping coefficients and shell properties. Beside the other factors, the local acoustic power is the principal parameter affecting microbubble behavior (Quaia 2005b). The local acoustic power depends on the output power of the ultrasound system, the

transmit frequency and the attenuation of the ultrasound beam with depth (Correas and others 2001). The output power is reflected by the mechanical index (MI) which originally measures the potential for mechanical damage to tissues exposed to intense ultrasound pulses. The MI also gives an indication of the likelihood of bubble destruction: the higher the MI, the greater its destructive properties (Averkiou and others 2003, Quaia 2005b). In clinical practice, the range for MI varies from 0.05 to 1.9. However, with the same MI the acoustic power may vary in different ultrasound systems (Quaia 2005b). Depending on the acoustic power, different interactions between the incident ultrasound beam and the microbubbles take place such as scattering, resonance and microbubble destruction.

Linear behavior of microbubbles - With very low acoustic power (MI <0.1), the microbubble radius does not change significantly over time and the bubble can be considered a static object. The scattering intensity increases with the intensity and the frequency of the incident ultrasound beam, and the number and radius of the microbubbles (Dalla Palma and Bertolotto 1999).

Resonance is another scattering mechanism observed at slightly greater acoustic power. Microbubbles respond to the positive and negative pressures of the sinusoidal sound wave by oscillation, changing their radius with time (contraction and expansion). At a particular frequency called fundamental or resonance frequency, these oscillations become resonant. The microbubbles oscillate synchronously with the incident ultrasound wave (Correas and others 2001, Leen and Horgan 2003). Since ultrasound contrast agents have microbubbles with a large size distribution, resonance does not occur at a single frequency but at a frequency range. Fortunately, the resonance frequency of microbubbles lies within the 2-15 MHz range, most frequently used for clinical diagnosis. Oscillation increases the scattering intensity because the average bubble's size is greater over time (Calliada and others 1998, Dalla Palma and Bertolotto 1999).

The linear effects of ultrasound contrast agents are utilized in the clinical setting by Doppler technologies, but rarely with grey-scale sonography because of the high attenuation coefficient. The signal to noise ratio drops rapidly with depth in the enhanced organ. Until recent advances in pulse sequence technology, discussed below, imaging of contrast agents for detecting tissue perfusion made use of exclusively the nonlinear effects.

Non-linear behavior of microbubbles - If the acoustic power of the ultrasound beam at the resonance frequency increases (intermediate MI: $0.1 < MI > 0.5$), the microbubbles will show a longer expansion than contraction phase and begin non-linear (non-sigmoidal shaped) oscillation (Correas and others 2001, Quaia 2005b). They start to emit the harmonics of the resonance frequency. The frequency of subharmonics is at half and that of higher harmonics is at multiples of the fundamental frequency. The second harmonic response is found at twice the fundamental frequency and usually has the highest intensity of the harmonic responses (Correas and others 2001). The theoretical advantage of the harmonic over the fundamental frequency is that only microbubbles resonate with harmonics while adjacent tissues do not resonate or their harmonic resonation is very little (Calliada and others 1998).

With further increase of the intensity of the ultrasound beam (high MI: $MI > 0.5$), the shell of microbubbles is disrupted and a transient strong non-linear echo is emitted (Correas and others 2001, Dalla Palma and Bertolotto 1999). This destruction modality is called stimulated acoustic emission or loss of correlation.

2.3.3. Classification of ultrasound contrast agents

Ultrasound contrast agents are grouped into 4 classes or generations (Bauer and Solbiati 2003). Several agents have been approved for the clinical use in humans. Although the approved indications are restricted, ultrasound contrast agents have also been used and tested widely for off-label indications in humans. So far, no contrast agent has been approved for veterinary use. In the present section, the most common ultrasound contrast agents are

discussed. It has to be mentioned though, that many more interesting ultrasound contrast agents are still in the phase of comprehensive testing and development.

At the beginning, contrast agents were hand-made, simply by agitating physiological saline solution prior to injection. In this way, free air microbubbles are produced and they represent the first class of contrast agents; however, because of their large size (>50 μm), they do not pass the capillary bed of the lungs. This hand-made agent is suitable for diagnosing an intra- or extracardiac right-to left shunt and is still used in veterinary echocardiography today (Kienle and Thomas 2002).

Microbubble persistence and stability was increased by two principal ways: bubble encapsulation with or without surfactants and selection of gases with low diffusion coefficient. This has led to the development of the first generation of transpulmonary contrast agents using air as the gas in the microbubbles covered by a shell. One of the most intensively investigated first generation ultrasound contrast agents is Levovist (Quaia 2005a). It is characterized by air microbubbles with a mean diameter of 2-3 μm covered by a galactose and palmitic acid shell. The shell provides increased microbubble stability and allows recirculation and capillary passage. Systemic enhancement of Doppler signals is produced over 1-5 min after i.v. bolus injection. After blood pool clearance, Levovist has been shown to have a late hepatosplenic-specific parenchymal phase with accumulation in the human liver and spleen up to 20 min after i.v. bolus injection (Quaia 2005b). Levovist only produces a clinically useful signal at medium to high acoustic pressure, and is simultaneously destroyed.

The second generation of ultrasound contrast agents uses insoluble gases to achieve better stabilization and lower diffusion of the microbubbles (Bauer and Solbiati 2003, Quaia 2005a). This increases survival of the microbubbles and therefore, the diagnostic window. Their half-life is longer than 5 min after intravenous bolus injection. One group of second generation ultrasound contrast agents is composed of perfluorocarbon-filled microbubbles, e.g. Definity, Imagent and Optison. Perfluorochemicals are inert compounds, which are immiscible with

water and can be injected intravenously if emulsified. Whereas Definity and Imagent have a phospholipid shell, Optison has a human albumin shell.

Another group of second generation ultrasound contrast agents is composed of sulphur hexafluoride-filled microbubbles, e.g. SonoVue. The advantages of sulphur hexafluoride-filled microbubbles are the prolonged stability in the vial (up to 6 hours) and the peripheral blood (half-life of 6 min), and the uniformity of their size, which improves backscattering and harmonic behavior at low acoustic power (Quaia 2005a). Similar to Levovist, SonoVue has also been shown to have a late hepatosplenic-specific parenchymal phase in humans (Leen and Horgan 2003, Lim and others 2004). SonoVue provides a non-linear response with production of a clinically useful signal at low acoustic pressures, and destruction of the microbubbles is limited.

Third generation agents use the stabilization of a hard shell (polymer shells) and contain either air or perfluorocarbons, resulting in a much longer persistence time (Leen and Horgan 2003).

2.3.4. Safety considerations

In general, ultrasound contrast agents are extremely safe and well tolerated. Guidelines for their clinical use in humans were published by the European Federation of Societies for Ultrasound in Medicine and Biology (Albrecht and others 2004). In humans, the incidence of side effects is low, predominantly minor in nature (headache, nausea) and self-limiting. Hypersensitivity or allergic events occur rarely. There is no evidence of nephrotoxicity or cardiotoxicity. However, there is the possibility of bioeffects such as microvascular rupture with the insonation of microbubbles. Therefore, caution is suggested when imaging the retina or the brain through the open fontanella. Further, premature ventricular contractions may occur when high mechanical index end systolic triggering is used in echocardiography but not with other applications. In general, long examination times and the use of contrast agents

within 24 hrs prior to extra-corporal shock wave therapy should be avoided. So far, no side effects have been reported with the use of Definity, Imagent, SonoVue and Levovist in dogs and cats (Nyman and others 2005, O'Brien and others 2004a, Rademacher and others 2005, Schärz and others 2005). Immune reactions have not been associated with the use of Optison in humans. Although there are reports of uncomplicated use of Optison in dogs and other small animals (Mattoon and others 2002), anaphylactic response related to the human albumin component has been reported in two dogs (Yamaya and others 2004). Severe but self-limited hypotension was observed, and the authors suggested mechanical ventilation and availability of emergency agents. Due to these complications and safer alternative contrast media, the use of Optison is considered contraindicated for clinical use in veterinary patients.

2.3.5. Imaging techniques

Imaging of contrast agents is more complex than mere addition of contrast media to conventional grey-scale and Doppler sequences. Manufacturers have long realized that bubble contrast agents are especially sensitive to the local deposition of sound energy. Sufficient energy (sound amplitude) must be provided to generate an adequate signal-to-noise ratio for agent detection, but not so high that bubble destruction occurs preventing real-time display of perfusion. This careful of energy balance includes adjustment of MI (output power), frame rate, number and location of focal zones, and transmitted frequency. Included in the pulse sequence adjustments are specific optimization of grey scale maps, frame averaging and edge enhancement. Technologies now exist to image contrast agents in both the fundamental (1^{st} harmonic) and harmonic (2^{nd} harmonic) frequencies by differing applications and manufacturers.

Fundamental imaging techniques - Ultrasound contrast agents can be used with conventional B-mode and Doppler sonography. Contrast agents cause significant enhancement of Doppler signals and have been used for many years for vascular studies. All conventional Doppler

modalities including color, power and spectral Doppler imaging, are very sensitive to the presence of microbubbles. Fundamental Doppler modes commonly use the stimulated acoustic emission modality with high MI settings. The contrast agent increases the Doppler signal, thereby increasing the sensitivity for low flow situations and detection of small vessels (Dalla Palma and others 1999). Conventional contrast-enhanced power Doppler ultrasound has been shown to be more sensitive for detecting low velocities and small parenchymal vessels than power Doppler alone or color Doppler with or without the use of a microbubble contrast agent (Bude and others 1994, Eriksson and others 1991). While color Doppler displays the change in the returning frequency to provide velocity and directional information, power Doppler records the amplitude (energy) of the reflected Doppler signal from moving blood cells. Color brightness is related to the number of moving cells, not the velocity. Because of the lack of angle dependence and the very low energy of random noise, higher gain settings may be used with power Doppler which increases sensitivity for flow detection (Rubin 1999). However, many imaging artifacts reduce the clinical benefits of contrast-enhanced Doppler ultrasound. These include high-intensity transient signals (flash), pseudo acceleration of the peak systolic velocity and blooming. To avoid interpretative errors, artifacts need to be recognized and the following settings should be adapted; reduction of color gain, persistence and MI, slower infusion of contrast agent, and increasing the wall filter and pulse repetition frequency (Quaia 2005b). Contrast-enhanced Doppler ultrasound is very sensitive to motion artifacts and is limited to the evaluation of more superficial structures or body regions less susceptible to respiratory and cardiac motions.

Grey-scale imaging in the fundamental (=transmitted) frequency of contrast agents has been historically limited for a number of reasons. Large dosages (0.08 ml/kg) of a second generation contrast agent (Imagent) were required for enhancement of vessels against the echogenicity of normal tissues (Bahr and others 2000). Little true perfusion imaging was achieved at these high dosages and the attenuation effects exceeded the enhancement benefits.

Contrast pulse sequence (CPS) is a new technology that processes the unique nonlinear fundamental and higher order harmonic signal (Phillips and Gardner 2004). By varying the phase and amplitude of multiple pulse interactions the contrast agent signal can be separated from the tissue signals. Because the effects of phase and amplitude on contrast agents are less frequency dependent than harmonic techniques, imaging is possible with higher frequency transducers (Cosgrove 2004). This allows more superficial structures or smaller patients, including smaller research rodents, to be imaged without loss of resolution inherent with the use of low frequency transducers in harmonic technologies. This CPS proprietary technology seems to hold great clinical and research promise.

Harmonic imaging techniques - Most of the commercial pulse sequence technologies have utilized the powerful emission of second-order harmonics for detection of contrast agents and, therefore, patterns of perfusion. As previously noted, contrast agents produce a disproportionately large amount of harmonic signal when insonated with frequencies approximating the resonant frequency. The most basic method for isolating the harmonic component is by filtering the returning signal (Correas and others 2001). Subtracting the fundamental (= transmitted bandwidth) portion from the returning signal should result in only the harmonic component being available for image formation. The advantage to this technology is that only a single pulse is transmitted for each line of image formed. However, the spectrum of transmitted frequencies overlaps with the returning harmonic signal and the filtering process must be carefully balanced between optimizing the harmonic signal against complete elimination of background tissue signal. More recent technologies have attempted to avoid this limitation by utilizing multiple pulse techniques. Inversion recovery is a 2-pulse technology that transmits sequential out-of-phase pulses and then adds the returning signal from each pulse (Averkiou and others 2003, Correas and others 2001). The tissue signals are identical and opposite and cancel each other when added together. The contrast agent signal is phase dependent (bubbles are resistant to compression) and the two returning agent harmonic

wave patterns are not mirror images and when added provide sufficient signal strength for image formation. This technology is used by multiple manufacturers and only suffers mildly from frame rate limitation of 2-signal pulse per imaging line.

Regardless of pulse sequence utilized, the system should be optimized for both low and high MI studies, as clinically indicated. Contrast enhancement in most parenchymal organs is divided into early and late phases. The late phase is approximately up to 20 minutes after introduction of the contrast material and after elimination of the majority of the blood pool portion of contrast agent. The remaining contrast agent is described variably within the sinusoids or reticuloendothelial system of the liver and spleen in humans (Quaia 2005a). A single high MI burst sequence produces a one time static picture of contrast agent distribution of the remaining contrast agent (Stewart and Sidhu 2006).

Low MI studies allow a real-time display of arteriolar perfusion and venous portions of early phase contrast imaging. As discussed later in the clinical applications section, the perfusion portion of the early phase imaging is especially important for determining the characteristic of liver nodules in the differential between malignant and benign nodules.

2.3.6. Image analysis

Qualitative image analysis - Contrast-enhanced color and power Doppler ultrasound

Contrast-enhanced color and power Doppler ultrasound images may be subjectively evaluated for vascularity (number of vessels per unit tissue volume), distribution of vessels within a lesion (vascular pattern) and vessel morphology. Subjective quantification of vascularity can be performed by using a score. Altered vessel morphology such as stenosis, occlusion, trifurcations, abnormal branching patterns and loop formation may also be assessed. All the criteria, either in combination or alone, have been used successfully in humans to differentiate benign from malignant lesions in various organs for example the lymph nodes (Tschammler and others 2002), the breast (Schroeder and others 2003) and the musculoskeletal system

(Bodner and others 2002). Contrast-enhanced power Doppler ultrasound has also been proven useful for the evaluation of rheumatoid arthritis in humans; it was able to detect changes in synovial perfusion after intra-articular steroid injection and therefore, helped with the evaluation of synovial inflammation and the assessment of a therapeutic response (Salaffi and others 2004).

Qualitative image analysis - Contrast harmonic ultrasound

Subjective assessment of regional contrast agent distribution in either the early or late phase provides the basis for characterization in the majority of clinical applications (Leen and Horgan 2003). For previously identified nodules, masses or abnormal organs (especially lymph nodes), characterization of the size, shape, number and location (=vascular pattern) of afferent vessels provides important information. During the perfusion portion of the early phase regional hypoperfusion can be detected and, when compared to timing of perfusion in the surrounding normal tissue, is vital for characterization of malignancy. All of this is performed in real-time and assessed subjectively. Similarly, occult malignant nodules can be detected by methodical scanning through an organ during the peak of the normal tissue perfusion. Regional hypoperfusion can be detected in cases of infarction, thrombosis or necrosis by comparison to more normal regions.

Quantitative image analysis - Contrast-enhanced color and power Doppler ultrasound

Most manufacturers provide online image analysis packages for post-imaging quantitative analysis. This is especially important for research applications. Different valuable computerized methods have been described in the literature for the quantification of vascularity assessed with contrast-enhanced Doppler ultrasound in human medicine (Cheng and others 1999, Fleischer and others 1999, Sehgal and others 2000) as well as in veterinary medicine (Nyman and others 2006a, Schärz and others 2005). Basically, a region of interest (ROI) is applied to the image, and the percentage of colored pixels within the ROI determines a vascularity index indicating the percentage area of the tumor occupied by blood vessels.

Taking the color level of each pixel, determined by the hue, saturation and brightness values, into account, a measure of blood volume or perfusion within the tissue may be derived.

Quantitative image analysis - Contrast harmonic ultrasound

With the onset of injection of an ultrasound contrast agent, time intensity curves can be generated over an appropriate time by applications of ROIs to assess perfusion within tissue volumes or individual vessels. The following quantitative hemodynamic indices are most commonly obtained: peak enhancement, time to peak enhancement, up-slope, down-slope and area under the curve (Nyman and others 2005, Ziegler and others 2003). By this method, baseline data for normal organ perfusion but also disease processes can be obtained. One caveat for determination of these indices is accounting for respiratory motion. Regardless of the MI, all imaging leads to destruction of contrast agent. Movement of the liver with respiration presents previously nonimaged liver with naive bubbles for analysis. Estimation of baseline and peak values is affected by patient motion. Time to absolute peak may introduce too many motion and bubble integrity variables in the analysis, falsely prolonging time-to-peak and lowering wash-in rate indices. A better method may be to calculate values of time to peak or indices of washout based on the times associated with 20% above baseline (as a better baseline value) and 90% (or other objective suboptimal value) of peak for the various indices, as appropriate for the organ and species (Ziegler and others 2003).

2.3.7. Veterinary clinical applications

Echocardiography - The first application of contrast ultrasound imaging was contrast-enhanced echocardiography. Ever since the discovery of the imaging advantages of bubbles in shaken saline injected intravenously, bubbles have proven to be clinically valuable. The two major clinical indications of contrast-enhanced echocardiography are improved edge detection for M-mode imaging and myocardial perfusion. Perfusion assessment is especially important for the diagnosis of cardiac infarction in human patients. But little attention has been paid to

this in veterinary medicine in spite of reports of myocardial infarction with feline hypertrophic cardiomyopathy (Van Vleet and others 1980) and other diseases with ischemic changes in dogs and cats (Driehuys and others 1998). Perfusion assessment of the left ventricular free wall and septum can be performed in dogs and cats with the same technology and transducers used for abdominal imaging, although this technology is not readily available on echocardiography systems.

Liver - The human liver is the most commonly studied abdominal organ for the use of ultrasound contrast agents. This is due to a combination of medical factors. These include the high prevalence and concurrent non-specific appearance of metastatic and benign nodules, hepatocellular carcinoma and cirrhosis in human patients. In veterinary medicine the liver is a very common site of metastasis of intra-abdominal primary neoplasia, especially hemangiosarcoma, and benign nodules are prevalent in older dogs.

The perfusion of liver in normal dogs has been described (Nyman and others 2005, Ziegler and others 2003). The mean time to peak perfusion (TTP) varied from 22.9 to 46.3 seconds. The variability may be related to dose of contrast, agent used, injection protocol and various machine settings. Additional variables include patient size and age. Anecdotal evidence indicates that portal circulation (TTP) is faster in smaller and younger patients. Neither study evaluated the effects of body weight or age on time to peak perfusion. Three young dogs (mean age = 4 months) with portosystemic shunts had very short TTP (7 seconds) (Salwei and others 2003). The effect of the portosystemic shunt was not separated from the additional effects of disparate age and body size from the previous studies. Additional studies would be helpful to determine some of the effects and to isolate the arterial from portal component. Comparing the arterial and portal venous components of hepatic blood flow may assist with the diagnosis of portosystemic shunts or portal hypertension.

The ultrasonographic diagnosis of malignancy of hepatic nodules is very challenging in small animals. There is no specific echo pattern for either benign or malignant nodules except

epithelial cysts. Nodules are commonly found in normal large older dogs, which are the same age and breed distribution for high risk for developing neoplastic lesions, including hemangiosarcoma. Detection of metastatic lesions from hemangiosarcoma by fundamental ultrasound is often difficult, being limited by nodule size and echogenicity, body size and concurrent diseases. A recent study indicated that contrast ultrasound is highly accurate for prediction of benign or malignant based on perfusion patterns (O'Brien and others 2004a). The results are similar to patterns determined for human patients with an additional pattern for malignant sarcoma. Benign solid regenerative nodules were isoechoic to the surrounding normal liver at peak normal liver perfusion. Hepatomas were more variable and had regions that were poorly perfused compared to surrounding liver. All malignant nodules were hypoechoic compared to the surrounding normal liver at peak normal liver perfusion. The study concluded that identification of tumor types was not possible although neuroendocrine tumors often had an intense rim enhancement pattern in the later portion of the early phase and hemangiosarcoma nodules (and some other sarcoma nodules) were very poorly perfused throughout the early phase with aberrant afferent vessels in the periphery of the nodule. Detection of small (1-2 mm) nodules was possible. A subsequent study showed improved detection of hemangiosarcoma hepatic nodules with detection of nodules not seen during grey-scale ultrasound (O'Brien, unpublished data). In at least one case the nodules seen on routine ultrasound were benign with contrast sonography while additional nodules, undetected with routine imaging, were malignant with contrast enhancement. These results demonstrate the utility of contrast-enhanced sonography for more accurate characterization of liver nodules and improved detection of malignant nodules.

Spleen –Little has been published on contrast ultrasound of splenic lesions in dogs. Infarctions in the spleen, similar to other organs, are easily identified (De Morais and O'Brien 2003). At the time, when the present study was undertaken, there was no perfusion or vascular characteristic which allowed discrimination between splenic masses. Smaller solid benign and

malignant masses may have similar perfusion patterns as liver nodules, but this is as yet undetermined. However, non-invasive differentiation of malignant splenic tumors from hematoma or benign nodules with contrast harmonic ultrasound would represent a valuable method since fine needle aspirates of splenic lesions may be non-diagnostic and tissue core biopsies may cause further bleeding. Contrast-enhanced Doppler may be of assistance for characterization of thromboembolic disease of larger vessels or torsion.

Lymph nodes - Normal lymph nodes have a primary afferent vessels which enter at the hilus, courses centrally and branches symmetrically along the length of the node. The angioarchitecture of malignant lymphomatous nodes was different from this normal pattern based on contrast sonography (Salwei and others 2004). In this study a high MI "angiographic" harmonic mode was used, although contrast-enhanced Doppler may also have been useful. The majority of peripheral lymphomatous nodes had aberrant vessels adjacent to or beneath the lymph node capsule, and deviation of the hilus.

Pancreas - In human patients most characterization of pancreatic disease is performed by contrast-enhanced computed tomography; contrast harmonic imaging of the pancreas has only been recently performed in a few studies. There was excellent correlation between regional necrosis, as identified by contrast CT, and duration of hospital stay and rates of mortality in human patients (Balthazar 2002). In another study contrast-enhanced dynamic CT showed enhancement of papillary adenocarcinomas whereas ductal adenocarcinomas did not enhance (Oshikawa and others 2002). These results were confirmed by contrast harmonic ultrasound in a recent study: all ductal adenocarcinomas were hypoechoic in contrast to the hyperechoic papillary adenocarcinomas (Takeshima and others 2005). However, inflammatory pancreatic masses also present as hypoechoic lesions and they are difficult to differentiate from ductal adenocarcinomas (Oshikawa and others 2002).

Contrast-enhanced color and power Doppler ultrasonography was shown a promising tool to assess feline pancreatic disease (Rademacher and others 2005). In comparison to normal cats,

vascularity and perfusion was significantly increased in cats with pancreatic diseases. However, whether inflammatory diseases can be differentiated from benign hyperplasia or neoplasia by the means of contrast-enhanced color and power Doppler ultrasonography could not yet be determined due to the small number of cases. Because of its depth limitations, contrast-enhanced color or power Doppler may not be used to assess the canine pancreas. Then, contrast harmonic ultrasound assessment of perfusion pattern may prove more useful.

Kidney - Renal imaging has some interesting differences from other organs in the abdomen. There is a very large arterial component with faster wash-in rates and stronger enhancement than in the liver in the same dog (Waller and others 2001). The kidney has two capillary beds with the cortical region enhancing earlier than the medullary region. Lesions that have been detected include infarction associated with thromboembolic disease, detection of otherwise isoechoic lesions, and characterization of masses (Nilsson 2004). Organ rejection often results in poor perfusion in the transplanted kidney of human cases. Contrast ultrasound perfusion imaging has been shown to be on par with Technetium 99m diethylenetriamine pentaacetic acid (^{99m}Tc-DTPA) scans for assessment of renal perfusion (Kim and others 2005). This technology may have interesting application in the expanding renal transplant field in veterinary medicine.

Superficial tumors - Like in humans, conventional contrast-enhanced power Doppler ultrasound has been shown to be more sensitive for detecting low velocities and small parenchymal vessels in superficial canine tumors than power Doppler alone or color Doppler with or without the use of a microbubble contrast agent (Schärz and others 2005). Contrast-enhanced color and power Doppler ultrasonography has shown benefit for the differentiation of superficial canine and feline tumors. In a recent study in dogs, vascularity and perfusion of oral squamous cell carcinomas was highest, whereas it was lowest for sarcomas and moderate for malignant oral melanomas (Laluhova and others 2004). However, although the differences were highly significant, the range of values overlapped between these histological groups.

Therefore, the authors concluded that the biological behavior may not only vary between but also within the same histology group. Further, the difference in vascularity and perfusion may play a significant role for the prognosis of various canine cancers. Similar results were found in cats. Cutaneous squamous cell carcinomas were highly vascularized and perfused (Ohlerth and others 2006), whereas vascularity and perfusion in fibrosarcomas was very low (Ohlerth, unpublished data).

3. Scientific objectives

Focal lesions of the spleen are common in dogs. Differentiation between the different splenic lesions especially between malignant and benign changes appears crucial because it influences treatment and prognosis. Splenic lesions are easily detected during routine abdominal ultrasonography, but their appearance usually does not allow a definitive diagnosis. Hyperplastic nodules, hematoma, abscess, primary or metastatic neoplasia, necrosis from vascular compromise, toxic conditions and inflammatory disorders can produce similar ultrasound findings. The type of malignant splenic neoplasia cannot be determined from its ultrasonographic appearance. Even cavitated lesions are not pathognomonic of hemangiosarcoma (Nyland and others 2002a). Moreover, hyperplastic nodules are not always observed with B-mode ultrasonography and diffuse changes are difficult to assess. In the liver, for example, improved detection of hepatic metastases of hemangiosarcoma was shown with contrast harmonic imaging with detection of nodules not seen during grey-scale ultrasound (O'Brien, unpublished data). Fine needle aspirates and tissue core biopsies of splenic lesions may be non-diagnostic because of blood dilution and tissue core biopsies may not be performed in every patient because of the risk of bleeding. CT and MRI represent promising new diagnostic tools in veterinary medicine; however, their availability is limited, examinations are expensive and patients need to be in general anesthesia. Therefore, non-invasive differentiation of malignant splenic tumors from hematoma or benign nodules with

contrast harmonic ultrasound would represent a valuable, cheap, easily performed and accessible method in veterinary medicine, similar to its use for the differentiation of liver lesions. Ultrasound is routinely performed in small animal medicine and widely available. The purpose of the present study was first, to determine normal perfusion dynamics in the canine spleen using contrast harmonic imaging. Second, criteria for the characterization of the vascular pattern and perfusion dynamics were established in clinical patients with cytologically or histologically confirmed splenic disease by the use of contrast harmonic imaging.

4. Materials and methods

4.1. Patient selection

A prospective study was performed from January 2005 until April 2006. Three groups of dogs were included in the study: 1. client-owned dogs which were presented with clinical signs unrelated to splenic diseases and which underwent a routine abdominal ultrasound examination; 2. healthy dogs owned by staff members of the Small Animal Department of the Vetsuisse Faculty of the University of Zürich, Switzerland, who volunteered to participate in the study; and 3. client-owned dogs which were presented with clinical signs suspicious for splenic disease and in which splenic abnormalities were detected ultrasonographically during a routine abdominal scan.

The study was approved by the Animal Ethics Council of the Kanton of Zürich. Owner consent was obtained for the contrast harmonic imaging study and fine needle aspiration of the spleen. Each patient underwent a physical examination. A blood analysis was undertaken to determine hematocrit, hemoglobin concentration and red blood cell count. Then B-mode ultrasound of the abdomen and finally, contrast harmonic imaging of the spleen was performed.

Spleens were considered normal if: a) conventional B-mode examination, contrast harmonic imaging and cytology of ultrasonographically guided fine needle aspirates of the spleen were unremarkable in clinically ill patients (patient group 1); or b) in clinically healthy dogs, no abnormalities were found with conventional B-mode and contrast harmonic imaging; ultrasonographically guided fine needle aspirates of the spleen were not taken in these dogs (patient group 2). Spleens were considered abnormal if focal or diffuse changes of the spleen were present with conventional B-mode ultrasound and/or contrast harmonic imaging. Then, lesions were confirmed with cytology of ultrasonographically guided fine needle aspirates or histology after surgery or necropsy (patient groups 1 and 3). Spleens were also considered abnormal if the conventional B-mode scan and the contrast harmonic imaging study were normal but cytology or histology suggested pathological changes of the spleen.

4.2. Ultrasonographic imaging

In the majority of cases, scanning of the dogs was performed using manual restraint only. However, uncooperative dogs or severely panting animals were sedated. After clipping and application of acoustic gel, a survey spleen scan was performed in every dog using conventional B-mode and a curvilinear array transducer at 5-8 MHz (ATL 5000, Philips AG, Zürich, Switzerland). For contrast harmonic imaging, a 2-5 MHz broadband curvilinear probe and pulse inversion harmonic imaging were used with a given machine preset (general imaging contrast, general). A moderate mechanical index was chosen first to depict a section of normal or diseased spleen as large as possible. Immediately prior to the injection of the contrast agent, the mechanical index was set to 0.08 while the probe remained in the same position. The contrast agent SonoVue (Bracco Research SA, Geneva, Switzerland) was administered intravenously through the cephalic vein as a rapid bolus at a dose rate of 0.03 ml/kg. This was followed by a rapid bolus of 5 ml saline. With the onset of injection, images were acquired for at least 120 seconds at a rate of one frame per second for the first 20

seconds and then one frame per 5 seconds. Images were stored as avi-files with the extended loop function of the ultrasound machine and then transferred to a personal computer. For subjective assessment of late phase uptake in the normal spleens, the organ was imaged for another 3 minutes and frozen images were taken every minute. Heart rate and blood pressure was monitored non-invasively in every dog during contrast harmonic imaging.

4.3. Image analysis

4.3.1. Conventional B-mode ultrasonography

The spleen was considered normal if the contours were smooth and regular, and the parenchyma appeared uniform, finely textured and more echogenic than the liver and the cortex of the left kidney (Nyland and others 2002a). Lesions of the spleen were assessed for their number (single, multiple), distribution (focal, diffuse), echogenicity (hypoechoic, hyperechoic, mixed), conspicuity (well defined, poorly defined), size, and presence of cavitary areas (yes, no). Presence of free abdominal fluid was also noted.

4.3.2. Contrast harmonic imaging

Subjective evaluation of the blood pool phase and late phase – In the normal spleens, images were assessed for the presence of an arterial and venous phase, and appearance of the parenchyma during the wash-in phase, peak enhancement and the wash-out phase. After the end of the blood pool phase spleens were also evaluated for persistent enhancement in the late phase up to 5 minutes after the injection of the contrast agent.

Splenic lesions were assessed for precontrast lesion conspicuity (well defined, poorly defined, invisible), lesion enhancement (yes, no), enhancement pattern (homogeneous, inhomogeneous, special patterns), and echogenicity in comparison to the surrounding normal spleen during the wash-in phase, peak enhancement and wash-out phase (hypoechoic,

isoechoic, hyperechoic). Any additional features were noted. Lesions were also evaluated for persistent enhancement during the late phase.

Quantitative computerized analysis of the blood pool phase – For this purpose, a commercial software program (QLAB Version 3.0, Philips AG, Zürich, Switzerland) was used. A region of interest (ROI), drawn as large as possible, was manually selected in every image avoiding inclusion of surrounding structures. A time-intensity curve was generated, and a given curve-fit, the gamma-variate, was applied. The following parameters were recorded: total time of examination, time to the initial rise, average derived peak intensity (PI), time to peak intensity calculated from the initial rise (TTP) and the area under the curve (AUC).

4.4. Statistical analysis

Statistics were performed with a computer software program (StatView® 5.0.1, SAS Institute Inc., Cary, NC, USA). Level of significance was $P < 0.05$.

5. Results

5.1. Patients

Twenty-four dogs were included in the study. The age ranged from 2 to 14 years (median, 6 years; mean, 7.2 years), and body weight ranged from 6.5 to 52 kg (median, 26.4 kg; mean, 25.6 kg). Five dogs had a body weight ≤ 15 kg: 3 Beagles, 1 Tibetian terrier and 1 Shetland sheep dog. Nineteen dogs of the following breeds had a body weight > 15 kg: mixed breed (n=9), terrier breeds (n=2), Golden Retriever (n=2), hunting dogs (n=2) and 1 Barsoi, Dalmatian and Belgian shepherd dog each. Eleven dogs were male and 13 female.

Mean hematocrit was 42.4% (median, 45%; minimum, 27%; maximum, 56%; reference range, 42-55%), mean hemoglobin was 13.9 g/dl (median, 14.9 g/dl; minimum, 9.6 g/dl; maximum, 16.6 g/dl; reference range, 14.4-19.1 g/dl) and mean red blood cell count was 6.1 x

10^6/µl (median, 6.5 x 10^6/µl; minimum, 4.1 x 10^6/µl; maximum, 7.5 x 10^6/µl; reference range, 6.1-8.1 x 10^6/µl).

Because of motion artifacts, one dog was sedated with butorphanol (Morphasol®, Gräub AG, Bern, Switzerland) at a dose of 0.1 mg/kg and another animal with buprenorphin (Temgesic®, ESSEX Chemie AG, 6005 Luzern, Switzerland) at a dose of 10 µg/kg. At the time of contrast harmonic imaging, mean heart rate was 110 beats/min (median, 97 beats/min; minimum, 60 beats/min; maximum, 168 beats/min; reference range, 60-130 beats/min). The systolic, diastolic, and mean arterial blood pressure at the time of contrast harmonic imaging ranged from 94 to 195 mmHg (median, 145 mmHg; mean, 138 mmHg), 57 to 129 mmHg (median, 82 mmHg; mean, 86 mmHg) and 71 to 140 mmHg (median, 103 mmHg; mean, 104 mmHg; reference range, 60-120 mmHg).

5.2. Ultrasonographic findings and pathological results

5.2.1. Conventional B-mode ultrasonography

Control group - Thirteen spleens were considered normal. Of these, 10 dogs (patient group 2) were clinically healthy and conventional B-mode examination of the spleen was unremarkable. Fine needle aspirates of the spleen were not available in these dogs. Three patients (patient group 1) suffered from diseases unrelated to the spleen (2 cutaneous mast cell tumors on the extremities, 1 brain tumor). Conventional B-mode examination and fine needle aspirates of their spleens were normal.

Diseased group – Eleven spleens were considered abnormal based on conventional B-mode findings and cytology or histology (patient groups 1 and 3). Nine spleens showed hypoechoic lesions with conventional B-mode. Distribution, number, conspicuity, echogenicity, size, and presence of cavitary areas varied among the lesions (Table 1). In one dog (dog 20), an enlarged spleen with a typical pattern (lacy pattern) most consistent with splenic torsion was found with a very hypoechoic parenchyma and multiple parallel echogenic lines. The hilar

perivenous hyperechoic triangle was also found. A small amount of anechoic free fluid was seen in the same dog, whereas a moderate amount of corpuscular free fluid was diagnosed in one hemangiosarcoma (dog 23). Cytological, histological or surgical diagnosis was hemangiosarcoma (n=3), hyperplasia of lymphatic tissue (n=4), extramedullary hematopoiesis (n=1), malignant lymphoma (n=1) and splenic torsion (n=1). The spleen of dog 18 was normal ultrasonographically; however, reactive hyperplasia was found cytologically.

5.2.2. Contrast harmonic imaging

In general, contrast harmonic imaging examinations were easy to perform. Side effects of the contrast agent were not observed in any dog. The feasibility of the examination was rarely limited and included respiratory motion and general motion; therefore, two dogs had to be sedated.

Subjective evaluation of the blood pool phase and late phase – Results of the subjective evaluation of contrast enhancement of the normal spleens (control group) were as follows: first, small splenic arteries radiating from the splenic hilus opacified rapidly after injection of the contrast agent. This was less well seen in small dogs. Subsequently, there was an inhomogeneous enhancement of the splenic parenchyma in all dogs. This phenomenon was more pronounced in some individuals than in others. Then, splenic parenchyma became homogeneously enhanced in every dog. Finally, a gradual decrease of opacification was observed. Persistent enhancement was neither seen during the blood pool phase nor during the late phase (Figure 1). Venous opacification was not a consistent finding and only identified in the hilar area.

Results of the subjective evaluation of 11 diseased spleens with contrast harmonic imaging are shown in Table 2. In general, lesions, in particular small lesions, were less well defined with harmonic imaging prior to contrast agent application than with conventional B-mode ultrasound. In all benign changes of the spleen (nodular or reactive hyperplasia,

extramedullary hematopoiesis) lesion enhancement was seen. In all cases with nodular hyperplasia except one, echogenicity was isoechoic to the surrounding spleen during the whole blood pool phase (wash-in phase, peak enhancement and wash-out phase) (Figure 2). In one very large nodular hyperplasia lesion, enhancement was most prominent during the wash-in phase, whereas at peak enhancement echogenicity was mildly hypoechoic in comparison to the surrounding spleen. All three hemangiosarcomas were hypoechoic at peak enhancement and well delineable (Figure 3). Large aberrant vessels and displacement of splenic arteries were seen in two hemangiosarcomas. In the malignant lymphoma, the diffuse small hypoechoic lesions in the spleen diagnosed with conventional B-mode could not be detected with contrast harmonic imaging before and during the contrast study (Figure 4). In the splenic torsion, no enhancement was observed at all (Figure 5).

Quantitative computerized analysis of the blood pool phase – Quantitative computerized analysis was possible in every spleen. In every normal spleen, a skewed bell-shaped curve was found during the blood pool phase (Figure 6). In the control group, mean baseline intensity was 0.55 dB (median, 0.39 dB; minimum, 0.00 dB; maximum, 2.1 dB) and mean intensity at the end of the blood pool phase was 2.9 dB (median, 2.24 dB; minimum, 0.75 dB; maximum, 7.1 dB) measured at a mean time of 142 s (median, 148 s; minimum, 99 s; maximum, 168 s). If the difference of the intensity at the end of the blood pool phase to baseline intensity was calculated, the mean was 2.34 dB (median, 1.66 dB; minimum, 0.27 dB; maximum, 7.05 dB); the difference was highly significant ($P = 0.009$; 95% confidence interval: 1.18, 3.51; paired t-test). Time to the initial rise in the control group showed a large range of values (mean, 15.2 s; median, 14.5 s; minimum, 5.8 s; maximum, 24.3 s).

For the control group, mean PI was 6.2 dB (median, 5.5 dB; minimum, 1.8 dB; maximum, 13.9 dB), mean TTP was 23.7 s (median, 20.1 s; minimum, 5.7 s; maximum, 49.9 s) and mean AUC was 479.6 dBs (median, 333.7 dBs; minimum, 27.2 dBs; maximum, 1575.7 dBs). If the control group was split into two body weight groups (≤15 kg and >15 kg body weight), the

median PI, TTP and AUC were lower for the smaller dogs (4.5, 12.6 and 153.5, respectively) than for the larger animals (6.0, 20.7 and 336.4, respectively). However, there was a large overlap of the ranges and differences were statistically not yet significant (P = 0.09, 0.16 and 0.16, respectively; Mann-Whitney U test).

To rule out a possible influence of the varying time of examination among all dogs, the quantitative analysis was also performed for a defined time of examination, e.g. 90 seconds or 100 seconds. No significant differences were found for the variables PI, TTP and AUC for the different examination times (paired t-test).

There was a highly significant difference between the control group and the diseased group concerning age and body weight. The diseased group was significantly older and more heavy than the control group (P = 0.0004 and 0.004, respectively; Mann-Whitney U test).

For the control and the diseased group, no significant association was found between the continuous variables hematocrit, hemoglobin concentration, red blood cell count, blood pressures, heart rate, age and the variables time of examination, time to the initial rise (t_0), average derived peak intensity (PI), time to peak intensity (TTP) and area under the curve (AUC) (correlation analysis). There was neither a significant difference between male and female dogs concerning the perfusion parameters (Mann-Whitney U test).

There was a highly significant positive correlation between AUC and PI (r = 0.96, 95% CI: 0.9, 0.98) as well as between AUC and TTP (r = 0.7, 95% CI: 0.37, 0.87). The correlation between PI and TTP was moderate (r = 0.56, 95% CI: 0.15, 0.81).

For the variables breed and sedation, a statistical analysis was not performed because of the low number per group.

If the benign lesions (hyperplastic nodules, extramedullary hematopoiesis) and the hemangiosarcomas were compared to the control group, median PI, TTP and AUC were the highest for the benign lesions (11.2 dB, 29.6 s and 933.5 dBs, respectively). The hemangiosarcomas showed lowest TTP and AUC (8.9 s and 149.4 dBs). However, the

number of cases per group was small and differences were statistically not significant (P = 0.08, 0.45 and 0.18 for PI, TTP and AUC, respectively; 1-way ANOVA and post-hoc Bonferroni-Dunn test).

6. Discussion

Morphological changes of the spleen are common in the dog. Diseases of the spleen may be clinically suspected; however, very often, they represent incidental findings during routine abdominal ultrasonography. They are easily detected, but their sonographic appearance usually does not allow a definitive diagnosis (Nyland and others 2002a). Although the final diagnosis was suggested in some cases in the present study, B-mode findings such as size of the lesion, presence of cavitary areas within the lesion or free abdominal fluid were not pathognomonic. For example, the fundamental sonographic appearance of the large hyperplastic nodule in dog 16 (Figure 2) and the hemangiosarcoma in dog 22 (Figure 3) was almost identical. Moreover, with B-mode ultrasound a false-negative diagnosis may be formulated although focal or diffuse benign or even malignant lesions may be present (Nyland and others 2002a). In dog 18, the spleen was normal ultrasonographically, but reactive hyperplasia was diagnosed cytologically. However, up to 10%, reactive hyperplasia may be considered normal.

Because of the high incidence of malignant neoplastic diseases in the canine spleen, differentiation especially between malignant and benign changes appears crucial because it influences treatment and prognosis. Ultrasound-guided fine needle aspirates or tissue core biopsies represent the method of choice to diagnose splenic disease. However, tissue-core biopsies are often avoided even by experienced specialists because of a high potential for hemorrhage. Lower quality samples may be attributed to red pulp congestion because of anesthesia or engorgement of the spleen. Aspirates and tissue-core biopsy specimens of complex splenic masses primarily containing fluid-filled cystic structures (e.g.

hemangiosarcomas) are less useful and often non-diagnostic because of blood dilution. Therefore, a negative aspirate does not rule out neoplasia. Moreover, repeated hemorrhages, necrosis, and organization may make hemangiosarcomas difficult to distinguish from longstanding infarction of the spleen or hematomas (Nyland and others 2002a). Whereas in clinically ill patients surgical excision and subsequent histological examination of the spleen often represent the most rational treatment, such an invasive procedure may be avoided in those dogs where a splenic lesion has been diagnosed incidentally. A non-invasive method with a high diagnostic accuracy appears a need in the diagnostic work-up of splenic diseases in the dog. In two recent studies, the usefulness of CT and MRI was demonstrated for the differentiation of benign and malignant splenic masses in the dog (Clifford and others 2004, Fife and others 2004). Nevertheless, the availability of these technologies is limited in veterinary medicine, examinations are expensive and patients need to be in general anesthesia. Contrast harmonic imaging represents a new ultrasonographic technology for the investigation of perfusion dynamics of tissue. It has been widely used in human medicine to classify spontaneous neoplastic lesions, mainly of the liver (Wilson and Burns 2001) but also of other organs, e.g. the spleen, lung and kidney (Catalano and others 2005, Gorg and others 2006, Nilsson 2004).

One of the goals of the present investigation was to evaluate subjectively the enhancement pattern of the normal canine spleen. Similar to the human spleen (Catalano and others 2005) small splenic arteries radiating from the splenic hilus opacified rapidly after injection of the contrast agent. Subsequently, there was an inhomogeneous enhancement of the splenic parenchyma in all dogs. This phenomenon was more pronounced in some individuals than in others. Then, splenic parenchyma became homogeneously enhanced in every dog. Finally, a gradual decrease of opacification was observed. Venous opacification was not a consistent finding and only identified in the hilar area. It appears important to know the normal patchy enhancement pattern at the beginning of the injection so that it is not wrongly interpreted as a

pathological condition. Subjectively, persistent enhancement was neither seen at the end of the blood pool phase nor during the late phase. However, the quantitative assessment of normal splenic perfusion showed that there was a significant difference of the intensity at the end of the blood pool phase to baseline intensity at the mean time of 142 s. We assume that this is most likely the result of a continuous wash-in of remaining contrast agent in the circulation rather than a true late phase enhancement as described in humans (Catalano and others 2005). In the normal human spleen, the total uptake of SonoVue did not decline after 5 minutes, in contrast to the liver or the kidney. The mean relative uptake even increased in the spleen after 5 minutes (Lim and others 2004). In our control group, no plateau phase was observed during the blood pool phase; instead, a skewed bell-shaped curve was seen during the blood pool phase in every dog. Moreover, persistent enhancement was also not seen during the late phase. In humans, persistent enhancement of the spleen with SonoVue is thought to represent tissue specific properties similar to the human liver. The underlying mechanism is not fully understood; in the liver, accumulation may be mediated by the reticuloendothelial system, e.g. the Kupffer cells, or microbubbles are entrapped in the liver sinusoids (Quaia 2005a). According to the results of our study, no evidence of tissue specificity in the spleen was observed in dogs.

In our control group, mean time to the initial rise was 15.2 s. Splenic artery opacification in humans is similar: 12 s (Catalano and others 2005). However, the range of the values was very large in our study group. Very likely, this may be due to a variable injection time with manual administration of the contrast agent. It may also reflect an influence of age, body weight, heart rate or blood pressure. However, in the present study no significant correlations were found.

A possible association of body weight and the perfusion parameters PI, TTP and AUC was found. In small dogs, ≤15 kg, values were lower, most likely indicating that overall perfusion is lower and the rate of inflow is faster than in larger dogs. We assume that this is due to the

different circulation (higher heart rate, lower overall blood volume in small dogs). However, results were not yet significant and should be verified in a larger study group. Then, normal splenic perfusion values should be calculated with respect to body weight.

Another purpose of the study was to investigate if perfusion patterns and dynamics differ in splenic lesions. Although the number of animals in the diseased group is rather low, some promising preliminary results were found. Subjectively, in all benign lesions (focal or diffuse) enhancement was observed and tissue echogenicity was iso- to mildly hypoechoic at peak enhancement in comparison to the normal spleen. This was in contrast to all hemangiosarcomas, where only very low enhancement was found. Although not yet significant, a similar result was found with quantitative assessment of perfusion. These results are similar to those found with CT (Fife and others 2004). On post contrast CT images, there was a significant difference in attenuation characteristics with nodular hyperplasia having the highest Hounsfield units, hematomas having intermediate Hounsfield units, and malignant masses having the lowest Hounsfield units values. The authors suggested that the low Hounsfield units for the hemangiosarcomas were due to the formation of large hematocysts or blood-filled cavities. The high degree of contrast enhancement of the hyperplastic nodules may have been related to the high degree of vascularity of hyperplastic lymphoid tissue. The underlying etiology of splenic hematomas in the dog was thought to explain the intermediate degree of contrast enhancement. In contrast to humans, splenic hematomas in the dog are often secondary to a primary disorder such as nodular hyperplasia. None of the dogs in their study had a history of trauma. However, the malignant masses in that study mainly included hemangiosarcomas and only one fibrosarcoma and one malignant fibrous histiocytoma. This is also a limitation of our study so far that only a few malignant splenic lesions have been investigated including only two different tumors. Other benign lesions such as hematomas were also not examined. The assessment of a larger variety of splenic diseases with contrast harmonic imaging will be a major goal for a future study. The results of the present study also

resemble the findings in the canine liver (O'Brien and others 2004a). Benign solid regenerative nodules were isoechoic to the surrounding normal liver at peak normal liver perfusion. Hepatomas were more variable and had regions that were poorly perfused compared to surrounding liver. All malignant nodules were hypoechoic compared to the surrounding normal liver at peak normal liver perfusion. Hemangiosarcoma nodules (and some other sarcoma nodules) were very poorly perfused throughout the early phase with aberrant afferent vessels in the periphery of the nodule.

One malignant lymphoma was also examined and showed homogenous overall enhancement. Lymphomas have been described as well perfused and vascularized neoplasms in humans (Tschammler and others 2002) as well as in dogs (Nyman and others 2006b). This tumor group appears important to be investigated in the future because it may not be differentiated from the benign splenic lesions solely based on the contrast harmonic imaging findings. Then, a combination of ultrasonographic B-mode and contrast harmonic imaging criteria may be more accurate.

No enhancement at all was observed in the splenic torsion. Although the B-mode findings were already highly suggestive of splenic torsion (lacy pattern, multiple parallel echogenic lines, hilar hyperechoic perivenous triangle, free abdominal fluid), it is well- known that these criteria are not pathognomonic (Mai 2006, Nyland and others 2002a). Contrast harmonic imaging may therefore help in diagnosing splenic torsion by diagnosing the overall absence of blood flow in the spleen.

Some limitations of the present study should be discussed. Contrast harmonic imaging was easy to perform in every dog in the present study. However, there are some limitations of this technology. Due to the low mechanical index (0.08) and the low frequency range (2-5 MHz), overall image resolution but also resolution in the near field is dramatically reduced in comparison with conventional B-mode. The problem may be partially overcome, as in the present study, by increasing the mechanical index prior to contrast injection to localize the

lesion. However, in small to very small lesions, in superficial structures and in small dogs, these factors represent clear limitations. Recently developed probes in the medium to higher frequency range revealed promising preliminary results and may represent a satisfying solution for this problem in the future (Cosgrove 2004).

Only in three dogs of the control group, a fine needle aspirate of the spleen was available confirming normal splenic tissue. Although B-mode ultrasound was normal in the other dogs, we cannot exclude a benign or even malignant disease. Even in the dogs with fine needle aspirate, a lesion could have been present in an area of the spleen other than the location where the sample was taken.

The diseased group was significantly older and heavier than the control group. This is most likely the consequence of the higher incidence of benign and malignant splenic diseases in older and large breed dogs. For a better comparison, more normal and heavier dogs should be included in the control group in the future.

Although no significant associations were found between perfusion and circulatory parameters e.g. heart rate and blood pressure in the present study, these factors appear crucial to be monitored during the contrast harmonic imaging procedure. Circulatory disorders may influence the perfusion of the normal as well as the diseased spleen. Two dogs had to be sedated for the sonographic examination. Drugs were used with mild cardiovascular effects; heart rate and blood pressure were normal in these dogs. However, in a contrast harmonic imaging study in the normal canine liver (Nyman and others 2005) using SonoVue, time to peak enhancement was significantly shorter in dogs anesthetized with propofol. Other perfusion parameters did not differ. Therefore, it is recommended considering the use of anesthetic drugs, if normal values are established.

In conclusion, the present study provides baseline data for the perfusion pattern and dynamics of the normal spleen in dogs. It may prove useful in the evaluation of dogs with diffuse or focal splenic disease. Preliminary results indicate subjectively or quantitatively

assessed differences in perfusion between normal, benign and malignant lesions of the canine spleen. Contrast harmonic imaging may represent a useful, non-invasive technology for the differentiation of splenic lesions. Further research must be done in a larger study group to determine the accuracy.

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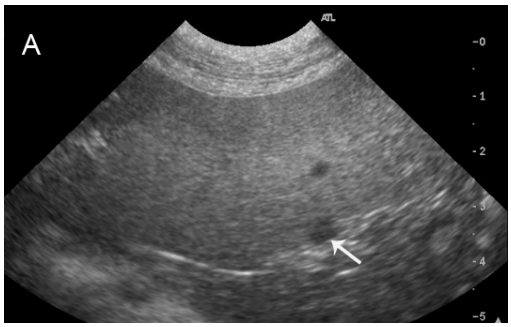
8. Appendix

Figure 1, A-G: Longitudinal images illustrating contrast enhancement of a normal spleen in a large breed dog (cranial is to the left, caudal to the right).

Figure A shows a B-mode image of the normal spleen. The arrow indicates a splenic vein at the hilus. Figure B represents an image taken just prior to the injection of the contrast agent. The probe and the machine presets are chosen for contrast harmonic imaging. Again, the arrow indicates a splenic vein at the hilus. In Figure C, opacified splenic arteries are seen radiating from the splenic hilus 13 seconds after injection of the contrast agent (white arrow). Subsequently, there is inhomogeneous enhancement of the parenchyma (25 seconds after injection, Figure D). Homogeneous enhancement occurs at peak enhancement (40 seconds after injection, Figure E). A gradual decrease of contrast agent opacification is seen thereafter (120 seconds after injection, Figure F). Subjectively, persistent enhancement is not seen during the late phase (5 minutes after injection, Figure G).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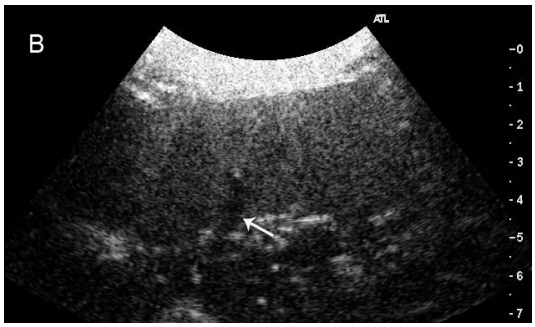
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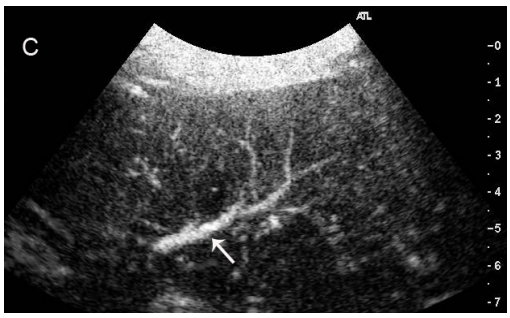
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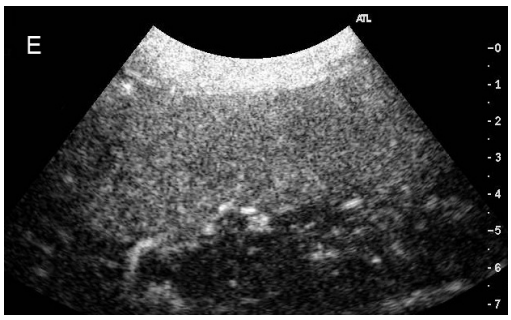
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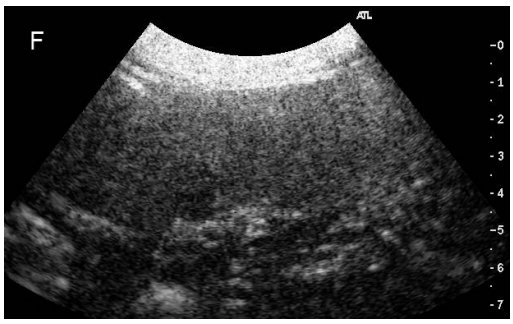
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G

Figure 2, A-E: Longitudinal images illustrating contrast enhancement in a large hyperplastic nodule of the spleen of dog 16 (cranial is to the left, caudal to the right).

In Figure A, the solitary hyperplastic nodule of mixed echogenicity is marked by calipers on a B-mode image. Caudal to the nodule, normal splenic tissue is seen. Figure B shows the nodule prior to injection of the contrast agent with the contrast harmonic imaging presets. During the wash-in phase (Figure C), the nodule enhances well but inhomogeneous and is mildly hyperechoic in comparison to the surrounding spleen. At peak enhancement (Figure D) the nodule shows an inhomogeneous enhancement pattern and is mildly hypoechoic in comparison to the adjacent spleen. During the wash-out phase (Figure E) the nodule becomes isoechoic.

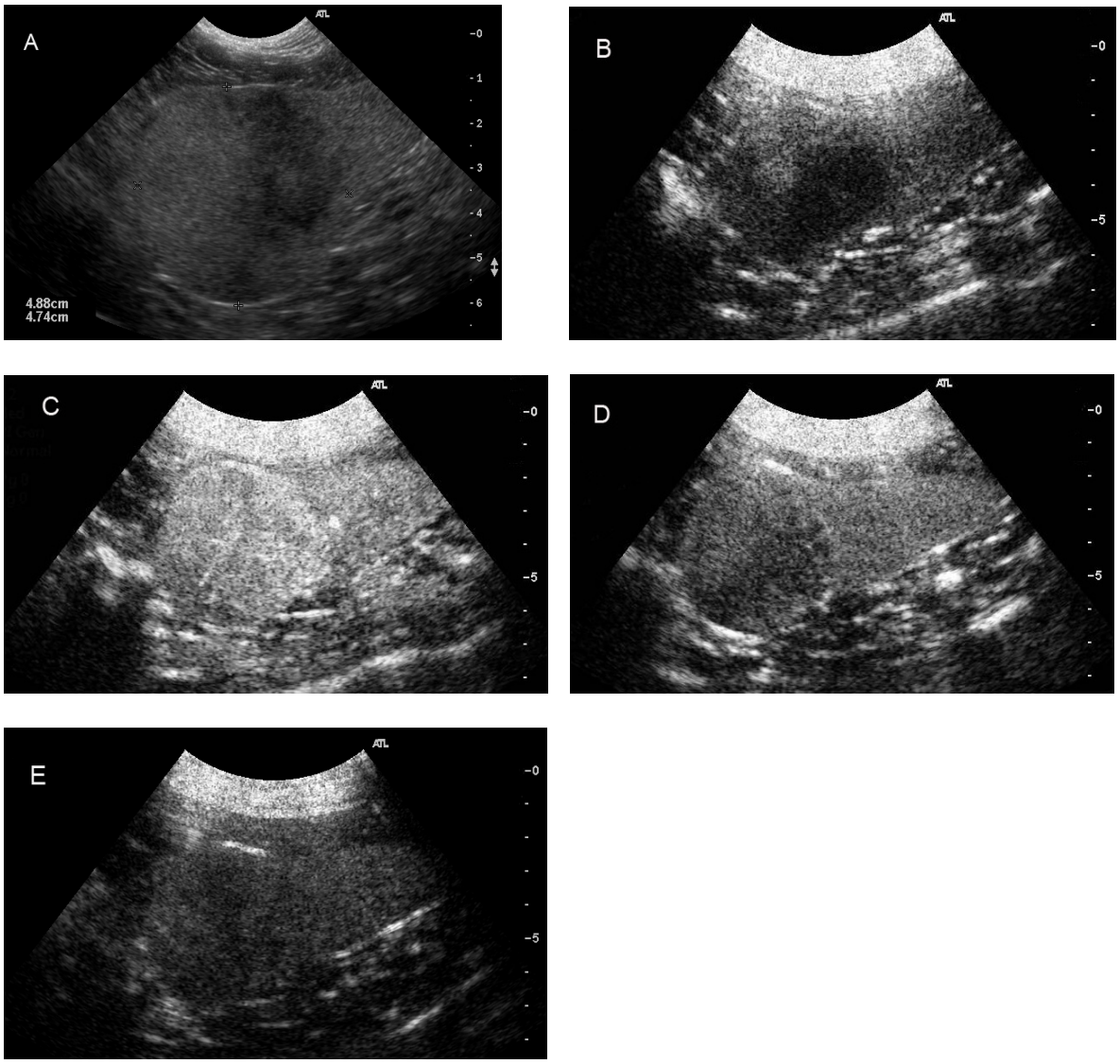

Figure 3, A-D: Longitudinal images illustrating contrast enhancement in a large splenic hemangiosarcoma of dog 22 (cranial is to the left, caudal to the right).

On the B-mode image (Figure A), the lesion in the tail of the spleen shows anechoic to almost cavitary and hyperechoic areas. Prior to contrast harmonic imaging (Figure B), the nodule appears inhomogeneous but cavitary areas may not be identified due to lower image resolution. At peak enhancement (Figure C), there is no overall uptake of the contrast agent in the nodule. Big aberrant vessels enhance in the center of the lesion (arrow). During the wash-out phase (Figure D) the nodule stays mostly hypoechoic in comparison to the adjacent spleen; the aberrant vessels are no longer seen.

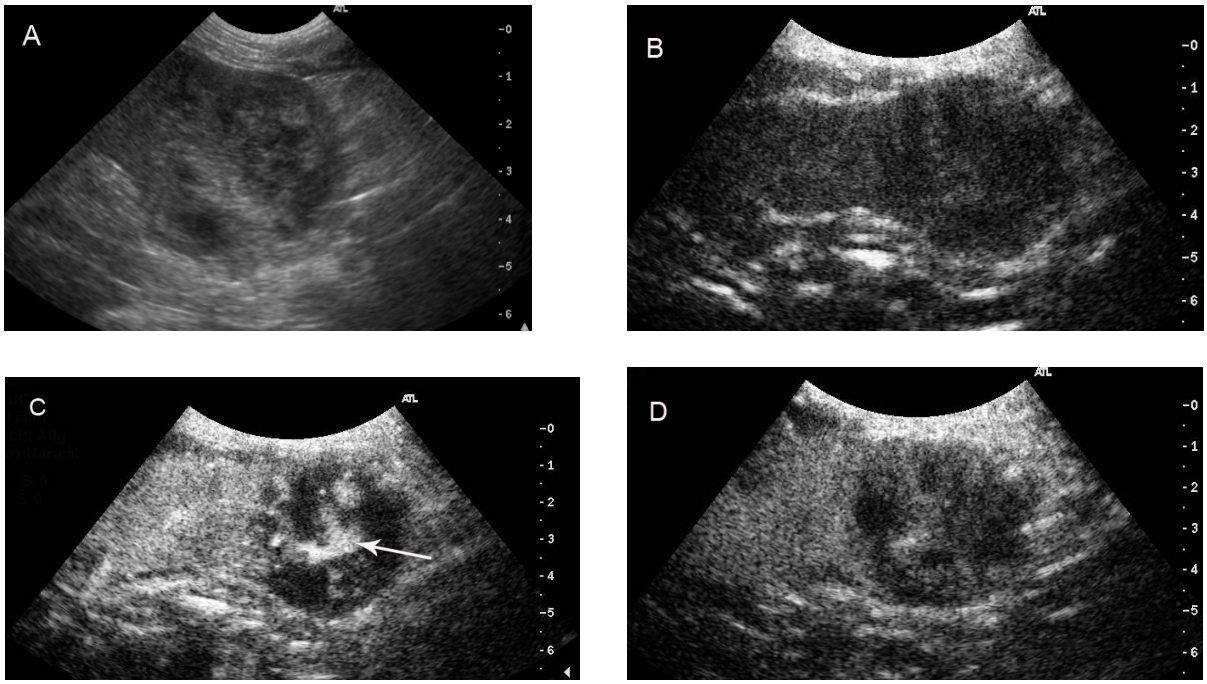

Figure 4, A-D: Longitudinal images illustrating contrast enhancement in a malignant lymphoma in the spleen of dog 21 (cranial is to the left, caudal to the right).

On the B-mode image (Figure A) very small hypoechoic lesions are identified throughout the spleen. Prior to contrast agent injection (Figure B), the lesions are not detected due to lower image resolution. At peak enhancement (Figure C) the lymphomatous spleen shows overall strong enhancement. During the wash-out phase the lesions are again not seen (Figure D).

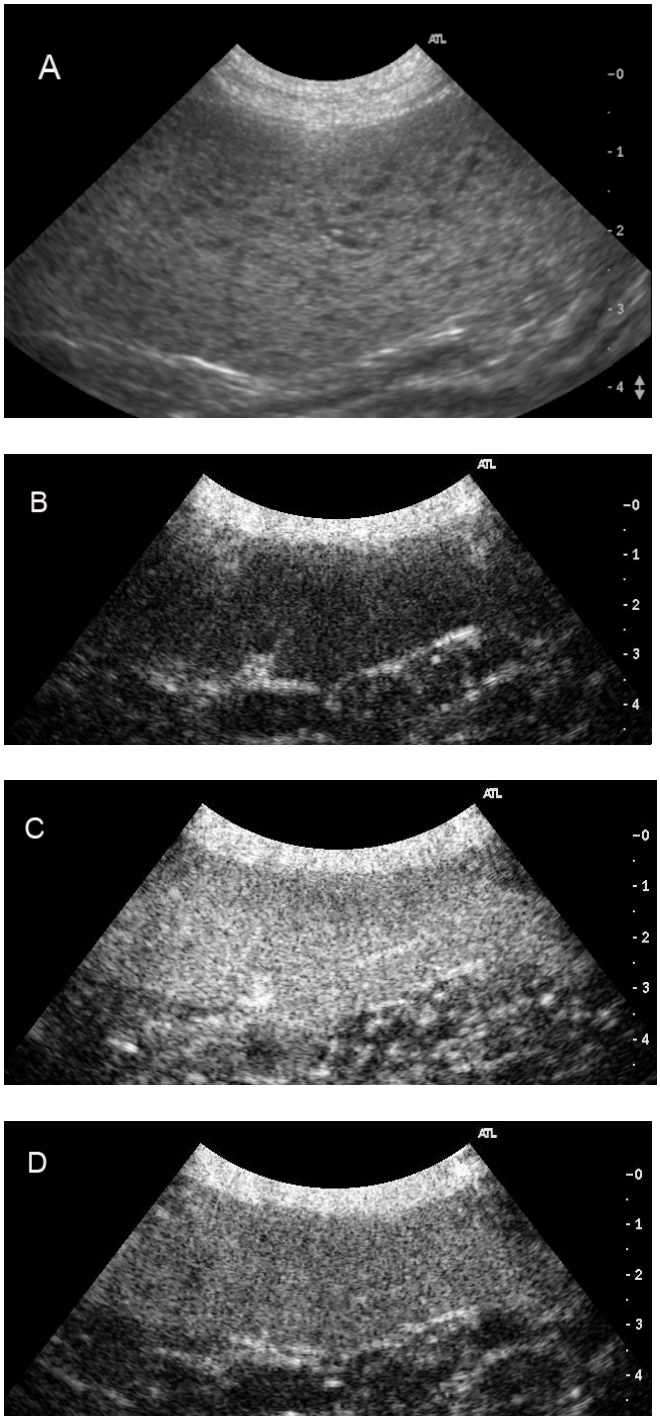

Figure 5, A-D: Longitudinal images illustrating contrast enhancement in a splenic torsion of dog 20 (cranial is to the left, caudal to the right).

On the B-mode image (Figure A) the spleen is markedly enlarged with diffuse anechoic areas and multiple parallel echogenic lines within the parenchyma. As a result of lacking blood flow, contrast harmonic imaging images do not show any enhancement prior to injection of the contrast agent (Figure B), at peak enhancement (Figure C) and during the wash-out phase (Figure D).

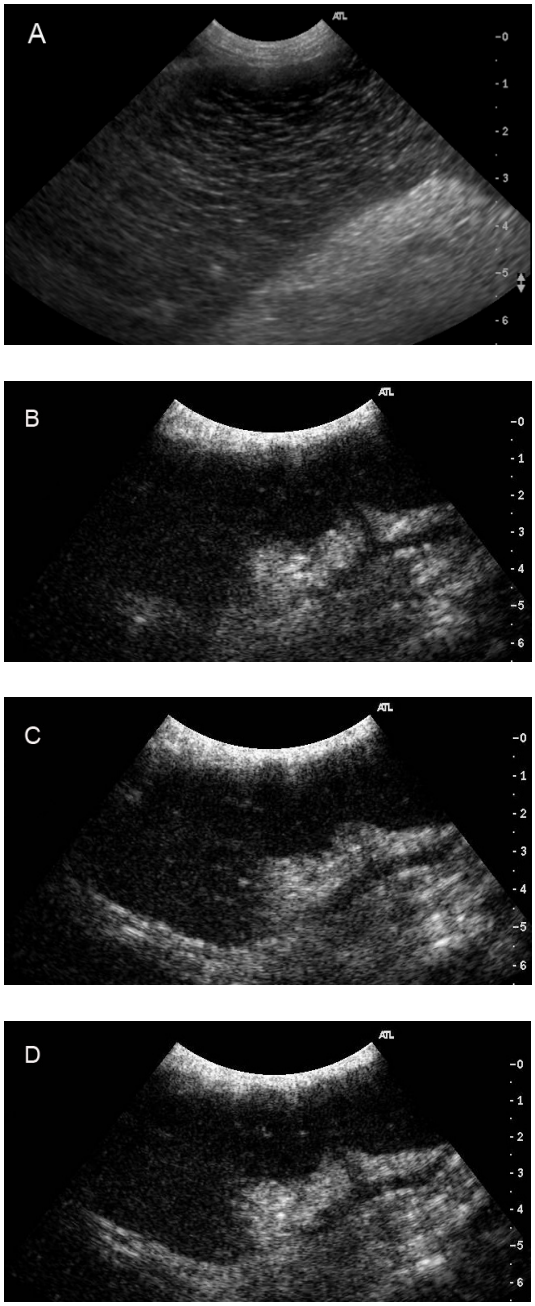

Figure 6: Time/intensity curve in a normal canine spleen (x-axis: time in seconds, y-axis: intensity in dezibel).

The oscillating curve represents the measured values during the examination. Regular deflections represent breathing artifacts due to normal in- and exspiration; single larger deflections represent motion artifacts. A given curve-fit, the gamma-variate, has been applied (continuous smooth line) resulting in a skewed bell-shaped curve during the blood pool phase. The following calculated parameters were derived from the curve: time to the initial rise (t_0), average derived peak intensity (PI), time to peak intensity calculated from the initial rise (TTP) and the area under the curve (AUC) (not shown).

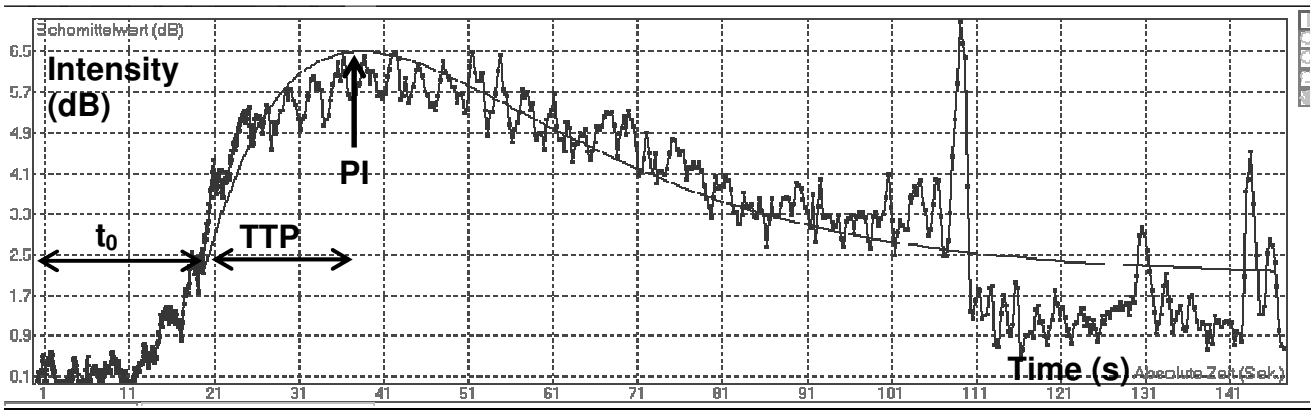

Table 1: Ultrasonographic findings with conventional B-mode in splenic lesions of 11 dogs

No	Patient	Clinical Diagnosis	Number, distribution	Echogenicity	Conspicuity	Maximum diameter♦	Cavitary areas	Biopsy Technique*	Pathological diagnosis
14	Mixed breed	testical tumor	multiple, diffuse	hypoechoic/ patchy spleen	poorly defined	n.m.	no	FNA	nodular hyperplasia
15	Tibetan terrier	anal gland tumor	single, diffuse	mixed	poorly defined	1 cm	yes	FNA	nodular hyperplasia
16	Labrador Retriever	oral fibrosarcoma	single, focal	mixed	well defined	4.8 cm	no	FNA	nodular hyperplasia
17	Golden Retriever	mastcell tumor	multiple, diffuse	hypoechoic/ patchy spleen	poorly defined	n.m.	no	FNA	reactive hyperplasia
18	Golden Retriever	brain tumor	normal B-mode of spleen					FNA	reactive hyperplasia
19	Hunting dog	enlarged spleen	multiple, diffuse	mixed/ patchy spleen	poorly defined	n.m.	no	FNA	extramedullary hematopoiesis
20	Mixed breed	weakness	diffuse	hypoechoic/ lacy pattern			no	HISTO	splenic torsion
21	Mixed breed	malignant lymphoma	multiple, diffuse	hypoechoic	well defined	≤ 0.5 cm	yes	FNA	malignant lymphoma
22	Airdale Terrier	fever, weakness	single, focal	hypoechoic	well defined	4 cm	no	HISTO	hemangiosarcoma
23	Mixed breed	collapse	single, focal	hypoechoic	well defined	1.5 cm	yes	HISTO	hemangiosarcoma
24	Mixed breed	fever, weakness	single, focal	hypoechoic	well defined	2.8 cm	yes	HISTO	hemangiosarcoma

♦ n.m.: not measurable

* FNA: cytological examination after ultrasound-guided fine needle aspirate, HISTO: histological examination after surgery or necropsy

Table 2: Subjective evaluation of 11 diseased spleens with contrast harmonic imaging

o	Patient	Pathological diagnosis	Conventional B-mode appearance, maximum diameter*	Precontrast lesion conspicuity	Lesion enhance-ment	Enhancement pattern, other features	Wash-in phase#	Peak enhancement#	Wash-out phase#
14	Mixed breed	nodular hyperplasia	patchy spleen, n.m.	invisible	yes	homogeneous	isoechoic	isoechoic	isoechoic
15	Tibetan terrier	nodular hyperplasia	mixed cavitary lesion, 1 cm	poorly defined	yes	homogeneous	isoechoic	isoechoic	isoechoic
16	Labrador Retriever	nodular hyperplasia	mixed lesion, 4.8 cm	well defined	yes	inhomogeneous	hyperechoic	mildly hypoechoic	isoechoic
17	Golden Retriever	reactive hyperplasia	patchy spleen, n.m.	invisible	yes	homogeneous	isoechoic	isoechoic	isoechoic
18	Golden Retriever	reactive hyperplasia	normal spleen		yes	homogeneous	isoechoic	isoechoic	isoechoic
19	Hunting dog	extramedullary hematopoiesis	patchy spleen, n.m.	invisible	yes	homogeneous	isoechoic	isoechoic	isoechoic
20	Mixed breed	splenic torsion	hypoechoic spleen, lacy pattern	invisible	no		hypoechoic	hypoechoic	hypoechoic
21	Mixed breed	malignant lymphoma	multiple hypoechoic lesions, ≤ 0.5 cm	invisible	no		isoechoic	isoechoic	isoechoic
22	Airdale Terrier	hemangiosarcoma	hypoechoic lesion, 4 cm	well defined	yes	inhomogeneous, large aberrant vessels	hypoechoic	hypoechoic	hypoechoic
23	Mixed breed	hemangiosarcoma	hypoechoic cavitary lesion, 1.5 cm	well defined	no		hypoechoic	hypoechoic	hypoechoic
24	Mixed breed	hemangiosarcoma	hypoechoic cavitary lesion, 2.8 cm	invisible	no	displaced splenic arteries	isoechoic	hypoechoic	isoechoic

*: n.m.: not measurable; #: echogenicity of the lesion in comparison to the surrounding spleen

Table 3: Results of the quantitative computerized analysis of the contrast harmonic imaging study in normal (n=13) and diseased spleens (n=11)

	Patient	**Group**	**Pathological diagnosis**	**Examination time [s]**	**t_0 [s]**	**PI [dB]**	**TTP [s]**	**AUC [dB·s]**
1	Dalmatian	Control group	not performed	147.29	19.71	4.41	18.51	222.00
2	Beagle		not performed	146.00	17.44	5.10	12.34	170.99
3	Beagle		not performed	138.00	21.82	1.77	5.67	27.24
4	Mixed breed		not performed	148.81	6.31	6.10	20.11	333.69
5	Mixed breed		not performed	150.64	24.29	5.50	49.88	745.85
6	Barsoi		not performed	99.24	7.12	12.79	36.45	1267.76
7	Shetland sheep dog		not performed	127.56	16.28	3.92	12.76	135.91
8	Staffordshire terrier		not performed	168.40	17.13	5.98	20.70	336.44
9	Beagle		not performed	152.72	16.56	5.63	39.44	603.06
10	Dutch shepherd dog		not performed	150.59	13.76	4.12	14.84	166.05
11	Mixed breed		normal spleen	111.00	15.24	7.67	26.02	542.25
12	Hunting dog		normal spleen	153.36	16.93	4.02	9.87	107.91
13	Mixed breed		normal spleen	156.64	5.79	13.85	41.86	1575.74
14	Mixed breed	Diseased group	nodular hyperplasia	108.06	12.00	13.41	32.87	1198.53
15	Tibetan terrier		nodular hyperplasia	138.89	4.22	14.06	26.36	1007.57
16	Labrador Retriever		nodular hyperplasia	155.00	7.54	29.22	35.18	2793.59
17	Golden Retriever		reactive hyperplasia	157.64	22.21	6.00	10.28	167.83
18	Golden Retriever		reactive hyperplasia	156.65	28.47	3.53	11.57	111.06
19	Hunting dog		extramedullary hematopoiesis	154.64	8.29	8.98	35.23	859.45
20	Mixed breed		splenic torsion	129.64	7.14	0.09	1.59	0.39
21	Mixed breed		malignant lymphoma	148.00	8.19	18.95	41.65	2145.49
22	Airdale Terrier		hemangiosarcoma	93.64	7.07	9.66	25.99	682.31
23	Mixed breed		hemangiosarcoma	145.94	21.56	4.57	6.88	85.36
24	Mixed breed		hemangiosarcoma	147.93	11.07	6.20	8.87	149.04

t_0: time to the initial rise; PI: average derived peak intensity; TTP: time to peak intensity calculated from the initial rise; AUC: area under the curve

9. Acknowledgements

An dieser Stelle möchte ich mich bei allen bedanken, die zum Gelingen dieser Arbeit beigetragen haben.

Mein besonderer Dank gilt:

Frau Prof. Dr. med. vet. B. Kaser-Hotz für die Möglichkeit, in der Bildgebenden Diagnostik und Radio-Onkologie mitzuarbeiten, und für die kompetente Beratung und tatkräftige Unterstützung,
Herrn Prof. Dr. med. vet. A. Pospischil für die Übernahme des Korreferates,
Dr. med. vet. B. Hauser für das Korrekturlesen der Arbeit und die wertvollen fachlichen Kommentare zum Thema,
Frau Dr. med. vet. S. Ohlerth für die Bereitstellung eines sehr interessanten Dissertationsthemas, für die wissenschaftliche Leitung, für die kompetente und geduldige Beratung und für die grosse Unterstützung und Motivation,
Frau Dr. phil. M. Roos für die Mithilfe bei der statistischen Auswertung,
meinen Arbeitskollegen, den **Mitarbeiterinnen** und **Mitarbeitern** der Bildgebenden Diagnostik und Radio-Onkologie der Vetsuisse-Fakultät Universität Zürich, für ihre Mithilfe und die freundliche Aufnahme in ihr Arbeitsteam,
und **meiner Familie** für die jederzeit liebevolle Unterstützung.

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